University of Alberta

Effects of Leukotrienes on Lung Development

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of **Master of Science**

Department of Physiology

Edmonton, Alberta
Spring 2004

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ISBN: 0-612-96519-8

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ABSTRACT

Hyperoxia (>95% O_2) inhibits alveolarization in newborn rats, a model that replicates many of the features of the human chronic newborn lung disease, bronchopulmonary dysplasia. Previous studies have suggested that leukotrienes (LTs) are mediators of this inhibition. The main focus of this study was to determine the effects of leukotrienes on lung development.

LTs are increased by hyperoxia and inhibition of their synthesis restored normal alveolarization. We hypothesized that exogenous LTs would replicate the effects of hyperoxia on the inhibition of alveolarization. As predicted LTB_4 and LTC_4 inhibited while, surprisingly, LTD_4 accelerated alveolarization.

To test the involvement of LTD_4 in normal alveolarization, the $CysLT_1$ receptor antagonist, MK-571 (25 μg/kg) was administered to rat pups. Morphometric analysis demonstrated that MK-571 arrested alveolarization to the same degree as hyperoxia, which indicates that LTD_4 is a mediator of normal alveolarization.

These data demonstrate that LTs are mediators that both arrest and accelerate alveolarization.

ACKNOWLEDGEMENTS

I would like to thank the following people for all of their help and encouragement over the past two years.

To **Dr. David Olson**: Thank you for your support and guidance throughout this project. I have learned a lot and have definetely grown from this experience.

To **Dr. John Greer** and **Dr. Redwan Moqbel**: Thank you for all of your suggestions and assistance.

To **Dean Zaragoza** and **Dr. Sheena Fang**: Thank you both for being there whenever I had a question- it meant a lot.

To **members of the Olson** and **Mitchell labs**: Thank you for supporting me throughout this project.

To **Robyn**, **Rachel**, and **Amanda**: Thank you for always being there when I needed a friend in Edmonton.

To **Rebecca S.**, **Julie**, **Erin**, **Gina**, and **all of my other friends**: Thank you for always being just a phone call away. Thank you for your friendship and support- I really appreciate it.

To **Jason**: I definetely could not have done it without you! Thank you for your patience, friendship, invaluable support and advice. At times you drove me crazy, but I do credit my sanity at the end of this experience to you!

To **Memere** and **Grandad**, **Kristina**, and **the rest of my family**: I love you all very much. Thank you for your unwavering support and encouragement. You gave me the confidence to believe that I could do anything.

To **Mom** and **Dad**: Without you this would have never been possible. You have supported me from day one, and even through the tough times I knew you were there for me. I respect you both, and have learned to be a better person from your example. I love you very much.

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LIST OF ABBREVIATIONS AND UNITS

The following are the abbreviations and units that were used in this thesis.

°C	degrees Celsius
5-HPETE	5-hydroxyeicosatetraenoic acid
12-LO	12-lipoxygenase
5-LO	5-lipoxygenase
AA	arachidonic acid
ASM	airway smooth muscle
BPD	bronchopulmonary dysplasia
BSA	bovine serum albumin
Ca^{2+}	calcium
CO_2	carbon dioxide
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CysLT	cysteinyl leukotriene
$CysLT_1$	cysteinyl leukotriene receptor 1
$CysLT_2$	cysteinyl leukotriene receptor 2
d	day
DdH_2O	double distilled water
ELISA	Enzyme-Linked Immunosorbent Assay
EtOH	ethanol
FGF	fibroblast growth factor

FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
g	grams
GGL	γ-glutamyl leukotrienase
GGT	γ-glutamyl transpeptidase
GPCR	G protein-coupled receptor
GSH	glutathione
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
HUVEC	human umbilical vein endothelial cells
IL	Interleukin
LT	leukotriene
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MAP	mitogen-activated protein
MK-0591	[3 Tert Butyl Thio (4 Chlorobenzyl) 5 (2 Quinolinylmethoxy) 2 Indoyl] 2,2 Dimethylpropionic acid
MK-571	3[2 Methoxy 4[(2 Methylbenzene sulfonyl) carbamoyl]]Benzyl Methyl 1h Indole 5 Carbamic Acid Cyclopentyl Ester
min	minutes

MMP	matrix metalloproteinase
MRP1	multidrug resistance-associated protein
n	number of replications
O_2	oxygen
O_2^-	superoxide anion
OH^-	hydroxyl
PBS	phosphate buffered saline
PDGF-A	platelet-derived growth factor
PN	postnatal
PPAR-α	peroxisome proliferator-activated receptor alpha
ROS	reactive oxygen species
s.c.	subcutaneous
SEM	standard error of the mean
SRS-A	slow reacting substance of anaphylaxis
TRITC	tetramethyl rhodamine isothiocyanate
VEGF	vascular endothelial growth factor
Wy-50295	Alpha Methyl 6 (2 Quinolylmethoxy) 2 Naphthylacetic Acid

Mathematical prefixes

c	centi (10^{-1})
m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})

CHAPTER 1. LITERATURE REVIEW

1.1 BACKGROUND

Leukotrienes (LTs) are lipid mediators that are derived from arachidonic acid and have been shown to be involved in pulmonary hypersensitivity reactions. Hyperoxia (>95% O_2) inhibits alveolarization, the final stage of lung development, in newborn rats (19). This is a model that replicates many of the features of the human chronic newborn lung disease, bronchopulmonary dysplasia (BPD). The studies in this thesis were undertaken to determine the roles of the individual LTs in lung development.

1.2 LUNG DEVELOPMENT

The lung is a complex organ that maintains a gas-exchange system to ensure that oxygen (O_2) is taken up and carbon dioxide (CO_2) is eliminated from the body. While it has no respiratory function during fetal life, it is essential for survival that it develops properly and matures for gas-exchange at birth (54). The lung must form in a specific fashion to create and maintain this system (101). This complex process occurs in five consecutive stages: the embryonic, pseudoglandular, canalicular, saccular and alveolar stages (Figure 1.1).

In the fourth week after conception (in humans) the primitive lung forms as an outpocket on the ventral side of the foregut (Figure 1.2). This is the embryonic stage of development and it lasts until the fifth to seventh week of pregnancy in humans (23), and from days 0-13 in the rat (26). The lung bud divides into two branches that begin to extend distally into the mesenchyme (27). These branches represent the early stages of the bronchi, and will continue to grow and they branch to create the airways of the lung

(23). They are lined by columnar epithelium that forms from the endoderm (23). This embryonic stage ends by d50 PN and the fetal one begins, which encompasses the other four stages (23).

The pseudoglandular stage now begins, and it is a stage that is characterized by rapid branching of the bronchi to the terminal bronchioles. It is called the pseudoglandular stage because the lung resembles an acinar gland throughout this period (23). As the airways form there is always a branch of the pulmonary vasculature tree that develops alongside it (54). At the proximal end of the lung tissue the airways are lined with columnar cells that begin to form cilia, and towards the periphery these cells become smaller and cuboidal (23). Throughout development there is a mass of undifferentiated cells at the ends of the developing airways, and as they branch these cells accompany them and differentiate (23). This mass consists of three times the amount of cells that are required by the airways at birth, which are precursor cells to the alveolar epithelium (25). It terminates, by week 17 in the human and embryonic day 18 in the rat ((26), with the formation of the acinus and the beginning of the canalicular stage (25)).

Throughout the canalicular stage the airways continue to branch and enlarge (25), and it is a stage that is characterized by extensive vascular growth. The capillary endothelium comes into close contact with the airway epithelium (22). Therefore, this is the beginning of the lung's gas-exchange system, and birth at this stage would result in survival, although assisted ventilation may be required. In this period the airway epithelium becomes squamous, which allows for efficient gas-exchange (23). By week 26 of gestation in humans, day 20 in rats, the acini have begun to subdivide into saccules and the lung has developed a spongy appearance (26). This indicates the beginning of the saccular stage, which continues until birth in humans (54). It is during this stage of lung development that rat pups are born.

Throughout the saccular stage there is both extensive branching and widening of airspaces in the lung periphery (23). The capillary network rearranges itself around this branching for further development of the gas-exchange system (25). Throughout this period the lung is preparing for birth with the development of the surfactant system and the entry of alveolar macrophage into the airspaces (23). The epithelium differentiates into type I and II pneumocytes (23). At the end of this stage, the parenchyma is composed of smooth-walled channels ending in terminal sacs (25).

The final period of branching morphogenesis is the alveolar stage; during which septation of the saccules and the formation of the alveoli occur. It is composed of two separate processes, alveogenesis and angiogenesis, which must occur together to ensure normal lung development. This stage is characterized by the formation of small buds (secondary septa) form along the terminal sac walls (primary septa) (25). Next, the secondary septa elongate and divide the saccules (11). For this to occur, two capillaries develop along each side of the septal bud which are separated by interstitium (25) (Figure 1.3). As the buds elongate, the capillaries fuses together to form a single capillary layer, which ends the growth of the secondary septa (25). This forms the alveoli and expands the surface area of the lung, which is important for the gas-exchange system of the lung. Formation of the alveoli demonstrate that the development of the pulmonary vasculature (angiogenesis) is directly related to the growth of the lung (alveogenesis) (11). The interalveolar septum is composed of the single-layer of capillaries along with a fibrous area of collagen and elastic fibers. It thins to maximize the contact between the airway epithelium and capillary endothelium for the gas-exchange system (24).

Alveolarization begins at 36 weeks gestation in humans and continues until 1-2 years after birth (d4-14 PN in rats (26)). The alveoli continue to enlarge until 8 years after birth (54) with over 80% of the alveoli forming postnatally (118). By the end of lung development there are 300 million alveoli are 250 µm in diameter (24). Type I pneumocytes are thin squamous cells that represent 93% of the alveolar epithelium (27). The remaining 7% are cuboidal surfactant-producing type II pneumocytes (27). There are also type III pneumocytes; however, these are rare and their function is unknown (27). The lung is composed of both non-parenchymal (bronchi, vessels and pleura) and parenchymal tissue (airspaces and septa). The airspaces represent over 80% of the parenchymal tissue (24).

The lungs of the preterm infants are different from term infants because they develop in external settings, and not *in utero* (145). They are born in the saccular stage of development and not the alveolar stage. Surfactant production has just begun in the saccular stage; therefore these infants are treated with artificial surfactant. While this aids in survival, it also causes an inhibition of septation. This results in decreased alveolarization and vascular development (78).

Currently it is not known which factors are involved in either normal or early lung maturation. It has been demonstrated that preterm birth causes infection and inflammation. The immune system has not yet developed (few alveolar macrophages), and the preterm lung is very susceptible to injury (78). This can be both helpful (factors released which promote early lung maturation), and harmful (factors released which cause lung injury) (78). Glucocorticoids, cytokines and oxygen exposure inhibit alveolarization (78) (Figure 1.4). Glucocorticoids are administered to preterm infants as anti-inflammatories; however, they inhibit septation and cause larger than normal airspaces (100). Retinoic acid is administered because it promotes alveolarization, and is administered to prevent the damage caused by the glucocorticoids (102).

1.3 VASCULAR DEVELOPMENT

There are three mechanisms that explain the development of the pulmonary vasculature. Vasculogenesis is the formation of new blood vessels from endothelial cells in the mesenchyme (74). Sprouting angiogenesis is the budding or branching of new vessels from pre-existing ones (74). Intussusceptive angiogenesis is a new concept concerning capillary growth. It was first characterized in the lung, and involves thin transcapillary tissue pillars, which increase in size and fuse with the capillary tissue (28). Development of both the airways and the vasculature must occur together because the inhibition of one will result in the inhibition of both (74). Vascular development is regulated by many factors, such as vascular endothelial growth factor (VEGF) and O_2; however, these mechanisms are not completely understood (97).

1.4 BRONCHOPULMONARY DYSPLASIA

Bronchopulmonary dysplasia (BPD) was initially described in 1967 as severe lung injury in preterm infants due to barotrauma and O_2 exposure caused by ventilation (109). This occurred in infants that were at the end of the saccular stage, and was characterized by lesions, fibrosis and enlarged airways (145). The new form of BPD, also known as chronic lung disease (77), is a progressive disorder that does not have the extensive histologic and radiologic symptoms associated with classic BPD (145). It occurs in infants at an earlier stage of lung development, usually in the canalicular stage, who do not have lung damage at birth; and is characterized by enlarged airspaces (99, 105). It is also caused by volutrauma, barotrauma and hyperoxia during mechanical ventilation, and can be stimulated by factors such as infection and inflammation (chorioamnionitis) (78).

BPD is a major cause of both morbidity and mortality in preterm infants (2, 53). Most are born before 28 weeks gestation in the late canalicular stage or the beginning of the saccular stage, and therefore the gas-exchange system has not fully developed (144). BPD frequently develops in very low birth weight infants (<1000 g) who exhibit little lung damage at birth (38, 67). The occurrence of BPD is indirectly proportional to the gestational age of the infant, with high frequency at <28 weeks (123). While the number of cases has not changed over time in relation to the amount of preterm births, improved technologies and treatments have caused an increase in the number of survivors (143).

BPD is characterized by inflammation, fibrosis, airway smooth muscle (ASM) hypertrophy, vascular abnormalities, and the progressive deterioration of lung function (40, 110). This can lead to the advanced disease, which includes bronchial hyperplasia, extensive edema, thickening of the basement membranes, and fibrosis with damage to the airways (145). The vascular system undergoes extensive remodeling to account for the stresses that are placed on it outside the womb, which includes dilated capillaries and capillaries in abnormal locations in the septa (11, 139). Morphologically BPD lungs have a reduced number of alveoli, which are enlarged and thick-walled (99, 105). Many of the symptoms, including airway hyperresponsiveness, chronic hypoxemia and airflow limitation, can persist into adolescence (9).

1.5 HYPEROXIA

High O_2 concentrations inhibit neonatal rat lung development by impairing septation (76, 103, 119). Prolonged O_2 exposure to both newborn and adult rat lungs cause interstitial and intra-alveolar edema, which allows cells and hemorrhage to enter the alveoli (32). After two weeks of hyperoxic exposure, newborn rat lungs exhibit morphologic changes (97, 121).

When preterm infants are placed on ventilators there is an increase in the pO_2 and barotrauma, which injures the lung. The lung is in direct contact with the O_2 administered and free radicals are produced, which causes toxicity to the lung tissue (145). They are produced from normal cellular functions (oxidation-reduction processes). The free radicals, or reactive oxygen species (ROS), include the hydroxyl anion (OH^-), the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The lung contains an antioxidant system to prevent the formation and damage of the ROS (34); however, preterm infants are born before this develops (61). Therefore, a greater number of ROS are formed (123).

The increase in ROS damages the endothelial cells (83, 97), and impairs their ability to repair themselves (90). The exact mechanism of this damage has not been established, although ROS cause widespread remodeling of the vasculature of the lung (121). It also causes edema fluid to leak into the airspaces (83), which can propagate the damaging effects. This capillary damage prevents the septation of the saccules into alveoli, and therefore decreases the surface area available for the gas-exchange system (121, 135).

ROS also behave as second messengers to promote the release of other mediators (123), since it is now believed that hyperoxic lung injury is a result of the activation of the inflammatory cascade and the release of inflammatory mediators (42, 51). The ROS cause a release in cytokines and other mediators that will propagate inflammation by attracting leukocytes to the lung. The arachidonic acid (AA) pathway that produces LTs has been shown to produce free radicals (134). One group of inflammatory mediators, the LTs, were the focus of this project.

1.6 PREVIOUS STUDIES FROM OUR LABORATORY

Our laboratory has developed a neonatal rat lung to study the effects of hyperoxia on this inhibition. More specifically, this model allows us to discover mediators that propagate the lung damage caused by ROS. The first studies were performed by Dr. Vera Boros, who measured the amount of LTs after hyperoxic exposure (95% O_2). She determined that the CysLTs were increased in newborn rat lungs after 10 days of hyperoxia as compared to controls (16). Dr. Boros also examined if this effect could be inhibited using specific antagonists to 5-LO (Wy-50295) and its activating protein FLAP (MK-0591), both of which are involved in the LT synthesis pathway (16). Both antagonists significantly reduced the amount of CysLTs in hyperoxia, and demonstrated that by inhibiting LTs, the inhibition of alveolarization was prevented (16). Dr. Gayle Hosford found that 5-LO, 5-lipoxygenase activating protein (FLAP) and LTB_4 levels increased in hyperoxia (62). These data suggest that LTs may be mediators in the hyperoxic effects on the newborn rat lung. Dr. Jacqueline Manji determined that a critical period exists for hyperoxic exposure mediated by the LTs (98). Exposure from postnatal days 1-4 and days 9-14 did not inhibit alveolarization; however, it was inhibited during exposure from days 4-9 (98).

Studies from other laboratories determined that the lipoxygenase products of AA metabolism are released in the presence of hyperoxia (73). Alveolar macrophages have been shown to release AA metabolites in the presence of hyperoxia to mediate the oxygen damage (132). Another study determined that preterm guinea pigs exposed to hyperoxia had increased levels of LTB_4 and pulmonary edema (117).

1.7 LEUKOTRIENES

The eicosanoids are a family of compounds derived from AA that behave as lipid mediators (48). These mediators include thromboxanes, prostaglandins and LTs (138), which were examined in these studies.

Until 1979 the LTs were believed to be a single mediator, and were known as the slow reacting substance of anaphylaxis (SRS-A) (91, 147). It was then determined that the SRS-A were actually a group of mediators that were named the LTs (125) (Figure 1.5). This name arose because they were released from leukocytes, and have three conjugated double bonds in a triene ring structure.

The LTs are formed through the 5-LO pathway of arachidonic acid metabolism (Figure 1.6). This pathway begins when cells are activated by mechanical, physical or chemical factors (12); and cytosolic PLA_2 ($cPLA_2$) is activated (149). Cytosolic PLA_2 ($cPLA_2$) then cleaves and oxidizes AA from membrane phospholipids. Mice that were lacking the gene for $cPLA_2$ did not produce LTs, which demonstrated its importance in the pathway (15).

5-LO is the key enzyme in the LT pathway, and depending on the cell type, is located either in the nucleus or the cytosol (48). Other enzymes also act on AA, such as COX-1, COX-2 and 12-LO; and 5-LO must compete with these to begin the LT pathway. FLAP is the activating protein for 5-LO, and is located on the nuclear envelope (147). In the presence of calcium (Ca^{2+}), it presents AA to 5-LO to convert it first to 5-hydroxyeicosatetranoic acid (5-HPETE), and then the unstable epoxide LTA_4 (48). LTA_4 is then hydrolyzed to LTA_4 hydrolase to form LTB_4 (47, 149).

LTB_4 causes superoxide anion production, chemotaxis and degranulation in leukocytes (71); and is involved in many inflammatory diseases, such as arthritis (149). It is chemotactic for both neutrophils and eosinophils, although more so for neutrophils (91). It has been shown to exert its effects through the recruitment of neutrophils (62). It also acts as a signaling agent that allows leukocytes to communicate (130).

LTA_4 can be metabolized through another pathway. It reacts with glutathione (GSH, γ-glutamyl-cysteinyl-glycine), through catalysis by LTC_4 synthase at the nuclear envelope to produce LTC_4 (48). This produces the CysLTs (peptido-LTs), which were named because of the peptide side chain located on the sixth carbon (58).

The membrane receptor MRP1 transports LTC_4 out of the cell for it to exert its actions. It is then metabolized to form LTD_4 and LTE_4 on the extracellular membrane when the amino acids on the peptide chain are cleaved off one by one (48, 91). LTC_4 is converted to LTD_4 by a γ-glutamyl transpeptidase (GGT) that cleaves the γ-glutamyl group from the peptide side chain (43, 113). LTD_4 is then cleaved by a membrane-bound dipeptidase to become LTE_4, which is 10- to 100-times less active than LTD_4.(52).

Recent studies have discovered an enzyme, γ-glutamyl leukotrienase (GGL), which also cleaves the glutamyl-glycine bond to convert LTC_4 to LTD_4. A knockout mouse deficient in GTT was produced which was able to cleave LTC_4 (31). This demonstrated that another enzyme, GGL, must be involved in the conversion of LTC_4 to LTD_4.

The CysLTs are produced in inflammatory cells such as basophils, eosinophils, mast cells and macrophages (48). These cells are all found in the pulmonary vasculature; therefore, LT synthesis occurs in these vessels (82). In the lung, alveolar macrophages are the main LT producers (62).

The LTs increase vascular permeability, mucous secretion, edema and bronchoconstriction the lung (13). They act as important mediators of the pulmonary vasculature as vasoconstrictors, and cause vascular leakage (70, 82). These symptoms are involved in the etiology of asthma and BPD (124). LTs also stimulate the migration of endothelial cells, indicating that they may be involved in angiogenesis (81). Another role of the LTs is to promote the recruitment of granulocytes into the airways to propagate the inflammatory response (89). However, the mechanisms of this chemoattraction are not known (12). The LTs cause a rise in cytosolic Ca^{2+} to initiate their signaling pathway (56). They are also 1000x more potent than histamine on ASM cells and have a longer period of action (124). They cause an increase in ASM, which changes the tone of the airways (59).

1.8 LEUKOTRIENE RECEPTORS

Before the LTs were identified in 1979, it was speculated that there were receptors for the SRS-A. This began with the discovery that FPL 55712 was an antagonist of the SRS-A (4), (125). LTs act at four different G protein-coupled receptors (GPCR); two for LTB_4: BLT_1 and BLT_2; and two for the CysLTs: $CysLT_1$ and $CysLT_2$ (48). CysLT antagonists were used on human pulmonary veins and ASMs to differentiate and name the two receptors (88). The CysLT antagonists ICI 198615, MK-571 and SKF 104353 blocked the contraction of the ASMs; however, they did not block the contractions of the vasculature (88). The receptors that were blocked were determined to be the $CysLT_1$ receptors, and those which were resistant were labeled the $CysLT_2$ receptors (88). These two receptors are responsible for the actions of LTC_4 and LTD_4, and are both glycosylated GPCRs (57).

GPCRs are the largest family of receptors, and are located on the plasma membrane (134). They are characterized by seven transmembrane regions, which makes them heptahelical receptors (112). Divalent cations improve their ability to bind with ligands, and binding is inhibited with sodium ions and GTP analogs (106).

The $CysLT_1$ receptor (also known as the HG55 receptor (94)) demonstrates different affinities to the LTs: $LTD_4 >> LTC_4 > LTE_4 >> LTB_4$, and is located on chromosome Xq13-q21 (94). The $CysLT_1$ receptor is 10-times more sensitive to LTD_4 than LTC_4, and 10-times more sensitive to LTC_4 than LTE_4 (94, 127).

The $CysLT_1$ receptor is expressed in the spleen, lung, pancreas and numerous other tissues as identified by northern blot analysis (46). It is also located on leukocytes such as eosinophils, monocytes, and macrophages (59). In the lung it is located on ASM cells, where it promotes bronchoconstriction (48). Almost all of the effects of the CysLTs, including mucus secretion and eosinophil recruitment, are mediated through the $CysLT_1$ receptor (111).

Once activated, the $CysLT_1$ binds to a heterotrimeric GTP-binding protein receptor, which causes an increase in intracellular Ca^{2+} (57, 64). A recent study demonstrated that the $CysLT_1$ receptor acts via two different G protein pathways (64). The first is a G_i protein pertussis toxin sensitive pathway that is stimulated by LTD_4 (124). G_i proteins cause the downstream activation of Ras, which is a low molecular weight G protein (93). It also causes actin reorganization in ASM by tyrosine phosphorylation (124). The other is a pertussis toxin insensitive G_q protein pathway (64). It stimulates an increase in intracellular Ca^{2+} concentration by first causing protein kinase $C\alpha$ to move from the cytosol to the membrane (64). It then acts with Raf-1 (mitogen-activated protein (MAP) kinase kinase kinase) to phosphorylate MAP kinase (64). It is speculated that the MAP kinase causes an increase in $cPLA_2$, which then produces more AA and LTs (64). This pathway involves many factors that have yet to be identified.

$CysLT_2$, like $CysLT_1$, has different affinities to the LTs: $LTD_4=LTC_4>>LTE_4>>LTB_4$, and is located on chromosome 13q14 (95). LTC_4 and LTD_4 are equally potent on this receptor; whereas LTE_4 acts as a partial agonist, and LTB_4 has little effect on the receptor (57). The human $CysLT_2$ was found to be 38% identical to $CysLT_1$ and 73% to rat $CysLT_2$ (57).

The $CysLT_2$ receptor was discovered from studies of human vascular tissue (75). It has now been studied pharmacologically and is involved in many of the effects the CysLTs have on the vasculature (46). $CysLT_2$ has been found in the spleen, heart and placenta through northern blot analysis (75). While $CysLT_1$ was mainly expressed in the ASM in the lung, $CysLT_2$ is most highly expressed in the lung macrophages (57). Both of the CysLT receptors are present in the lung on the endothelium (7). $CysLT_2$ appears to be involved in eosinophil recruitment (75). The binding of ligand to these receptors has recently been shown to be important when the lung is exposed to allergens (122). It produces Ca^{2+} mobilization in cells in the same manner as $CysLT_1$; however, the signal transduction pathway of the $CysLT_2$ has not yet been established (127, 133).

The two receptors are expressed on different tissues, and may have different functions in mediating the effects of LTs (69). The activation of the CysLT receptors releases mediators that propagate the effects of the CysLTs (5). LTD_4 causes a release of AA in cells, which then produces more inflammatory mediators (35). Products of the cyclooxygenase pathway of AA (prostaglandins) also mediate the effects of the CysLTs (37).

One study demonstrated that LTD_4 activated both $CysLT_1$ and $CysLT_2$, whereas LTC_4 acted on $CysLT_2$ (6). The CysLTs potentially act in an autocrine or paracrine fashion to exacerbate their damage on the lung (46). The two receptors may act together; for example, communication between the receptors could promote interactions between cells (91).

CysLT receptors are up-regulated by various cytokines. In HL-60 eosinophils, interleukin (IL)-5 increased the mRNA of the $CysLT_1$ receptor (136). It was also determined that these cells secreted IL-5, which acted on the receptor in an autocrine fashion (116). IL-4 and IL-13 stimulate the expression of $CysLT_1$ on monocytes and macrophages (137). Interferon (IFN)-y stimulated the expression of both CysLT receptors on ASM cells (3).

The receptors for LTB_4 are different from the CysLT receptors, and there is no evidence of crossreactivity (108). There are two LTB_4 cell surface GPCR receptors with similar signaling pathways: the high affinity BLT_1 and the low affinity BLT_2 (148), (150). BLT receptors have a 26-28% amino acid identity to CysLT receptors, which demonstrates a distant evolutionary association (127). The main action of LTB_4 is the chemotaxis of neutrophils, therefore BLT expression is highest on neutrophils; however, it has also been detected on other leukocytes, with the exception of macrophages and monocytes (66).

LTB_4 is the only LT with a dual receptor system, since it acts on the nuclear envelope through a peroxisome proliferator-activated receptor alpha (PPAR-α) nuclear receptor (48). This transcription factor activates the transcription of genes that terminate inflammation (41).

1.9 LEUKOTRIENE ANTAGONISTS

LTs are important mediators in both allergic and inflammatory diseases of the lung, because they are involved in both inflammation and bronchoconstriction (108, 131). They are important in asthma because it has been determined that after antigen administration to asthmatics their levels are increased (79). Also, hyperreactivity has been associated with the release of the CysLTs (65). Drugs that inhibit the CysLTs have been created as a new treatment method for asthma. Clinical trials using both 5-LO inhibitors and CysLT receptor antagonists have demonstrated improvements in the symptoms of chronic asthma (72, 120). Anti-LTs are as effective as anti-inflammatories in asthma, and inhibit both the early and late responses to allergens by acting together with histamine (49, 120, 131). LT antagonists are useful in treating both pediatric and adult asthmatics (12). The $CysLT_1$ receptor is involved in asthma (87), and there are many $CysLT_1$ antagonists that have been used to treat it, such as zafirlukast®, pranlukast®, and montelukast® (1, 57, 114).

MK-571 inhibits LTD_4 and not LTC_4, which demonstrates that it is an effective $CysLT_1$ antagonist (80). It is a lipophilic agent that is a very potent orally active antagonist (18, 80). Clinically it is well tolerated, has a fast onset and takes effect 24 h after administration (79). One study demonstrated that MK-571 inhibited vascular permeability, bronchial hyperreactivity and eosinophil infiltration (13).

The $CysLT_1$ receptor is sensitive to all of the current CysLT antagonists; however, most are ineffective at blocking $CysLT_2$ (133). Only Bay u9773, an analogue of LTE_4 antagonizes $CysLT_2$, along with $CysLT_1$. It is currently the only antagonist available to characterize this receptor (7, 57).

1.10 ANIMAL MODELS

The final stage of lung development, alveolarization, was the focus of these studies. It occurs postnatally in humans, and models have been created in animals whose lungs develop in a similar pattern. In humans, alveolarization begins before birth, whereas the rat is in the saccular stage of lung development at birth (25). In the rat model, alveolarization occurs from d4-14 PN, and is a period of rapid alveolar formation (140).

Impaired lung development can be caused in experimental animals with hypoxia (103), glucocorticoids (100), and hyperoxia (14, 78). In the current studies hyperoxia was used to replicate the O_2 damage induced by ventilation in preterm infants with BPD.

Various models have demonstrated that O_2 exposure can cause lung injuries that are similar to BPD. Newborn rats are a useful model because when they were exposed to hyperoxia, their lung morphology became similar to BPD lungs (119). This included enlarged airspaces and increased capillaries in the interalveolar septa. In rat pups, the alveolar capillaries were decreased after six days of hyperoxic exposure (121). The hyperoxic rat lung model has also been used to determine which growth factors are involved in O_2 toxicity (53).

Many studies have used primates because their lung development is very similar to humans. Studies using ventilated premature baboons demonstrated permanent alveolar loss by impairing of alveolarization in early development (2, 33). There were also abnormalities in these baboons which mimicked BPD, such as edema, airway thickening and fibrosis (33). To date, this is the best large animal model to use for BPD studies (53). However, it is very difficult to maintain a baboon colony. They have a long gestational period, and are expensive to house, since a neonatal ICU must be constructed to maintain the preterm baboons. Our laboratory has found the rat model to be the most feasible for our studies.

Other rodent models, such as mice, have also been established to examine the effects of hyperoxia. Bonikos *et al.* inhibited lung development by administering 100% O_2 to newborn mice (14). Another study exposed newborn mice to 85% O_2, and it produced an inflammatory response and inhibited septation (30). Mice have the same period of alveolarization as the rat (d4-14 PN) (17), and are a good model to use because they can be genetically manipulated.

Various knockout mice have been developed to determine the growth factors that were involved with lung development. For example, mice lacking the gene for VEGF did not survive past the embryonic period, which demonstrated that VEGF is an important factor in pulmonary vascular development (30). It also adds support to the concept that lung development depends on the airways and the vasculature developing together. If one is inhibited, then they will both cease to form.

Studies have also looked specifically at the effects of growth factors on alveolarization. One study demonstrated that the development of alveolar smooth muscle was impaired in knockouts for PDGF-A (platelet-derived growth factor) (92). Another discovered that secondary septal formation was inhibited in mice lacking the gene for the receptor for fibroblast growth factor (FGF)-3 and FGF-4, FGF receptor (FGFR)-3 and FGFR-4 (146).

A 5-lipoxygenase (5-LO) knockout mouse was established to determine the effects of leukotrienes (LTs) on infection (10). They found that these mice had a weakened antibacterial host defense system in the alveolar macrophages, which could be strengthened by exogenous LTB_4 (10). Another study used this model to demonstrate that LTB_4 was involved in the recruitment of neutrophils for phagocytosis in the lung (96). A knockout mouse was created for the multidrug resistant protein 1 (MRP1), which is the protein that transports LTC_4 from the cytoplasm to the extracellular environment, where it can be cleaved into LTD_4 (129). It was determined that increased levels of LTC_4 in the cytoplasm caused an inhibition of LTC_4 synthase in the cytoplasm (129). This was not competing with LTA_4 hydrolase for LTA_4, and there were increased amounts of LTB_4 in the lung, which aided in recruiting neutrophils to the lung to combat pneumonia (129). Unfortunately, none of these studies looked at these mice during alveolarization.

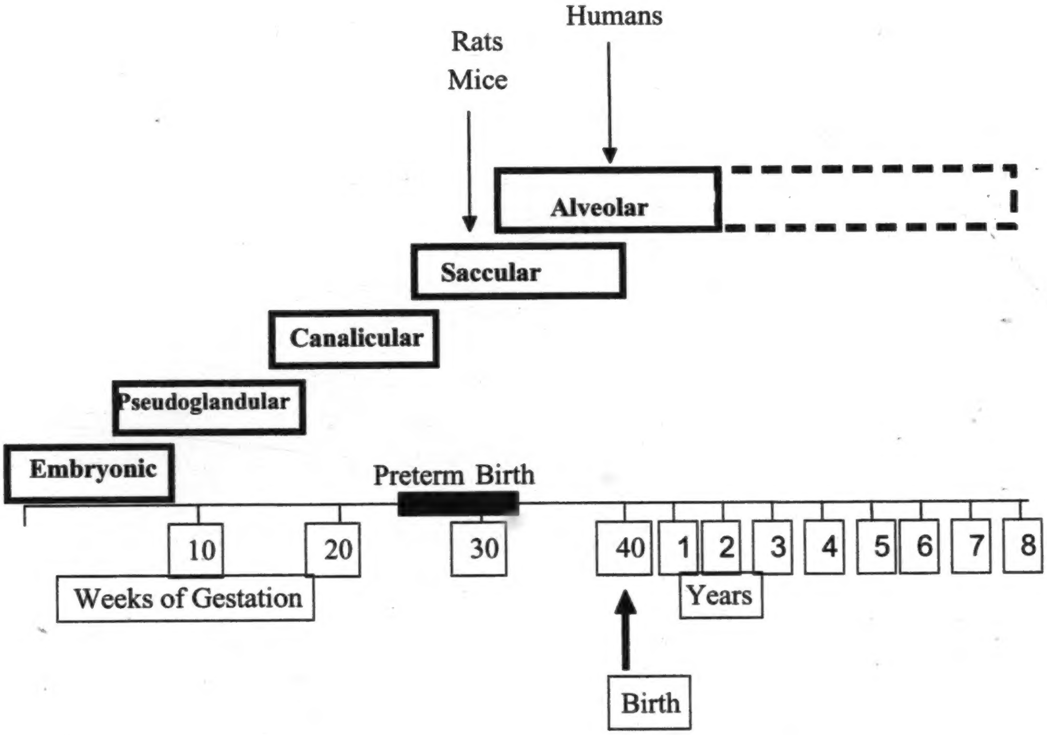

Figure 1.1. Lung development in various species.

This figure shows the five stages of lung development, and when they occur in human development. The period of preterm birth is highlighted. Alveolarization occurs from d4-14 PN in rats, which is a useful model for study of this stage of lung development. *Modified from Zeltner TB and Burri PH. The postnatal development and growth of the human lung. II. Morphology. Respir Physiol 67: 269-282, 1987* (54).

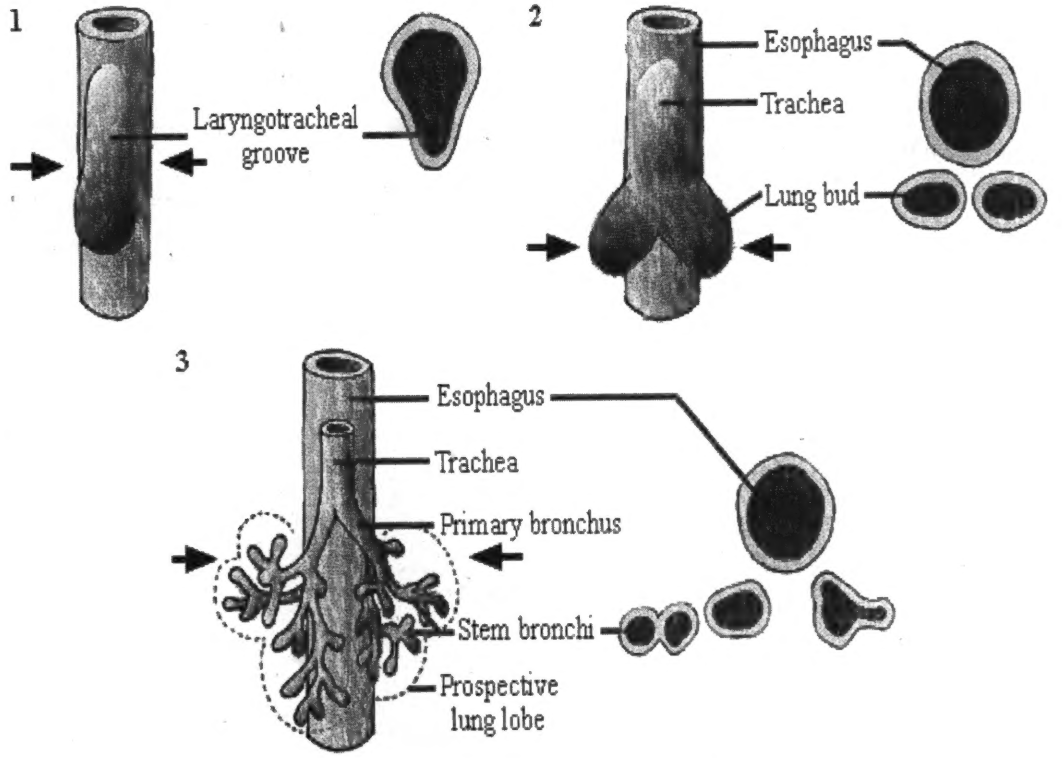

Figure 1.2. Embryonic development of the lung.

1: Ventral outpocketing of the gut and development of the lung bud *2*: Splitting of the lung bud into two branches. *3*: Branching and elongation of the airways. *Modified from http://www.uoguelph.ca/zoology/devobio/210labs/lung1.html.*

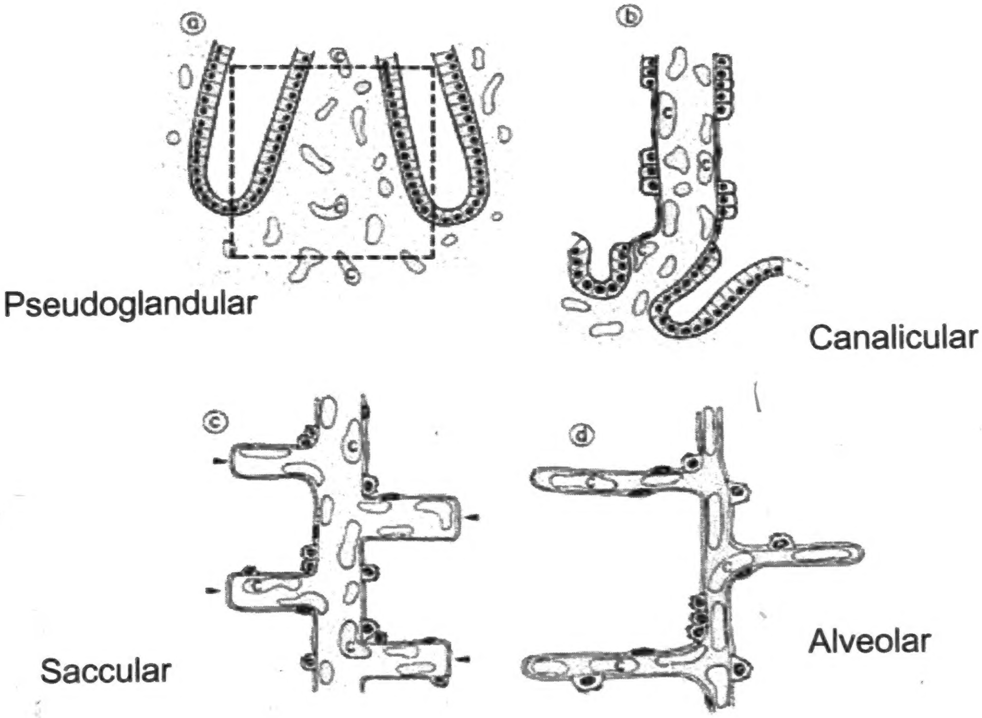

Figure 1.3. Development of the pulmonary vasculature.

A: Capillaries in the mesenchyme arranges itself around the developing airways. *B:* The capillaries arrange into the double capillary system, which is separated by interstitium. *C:* The secondary septa begin to bud, and contain the double capillaries. *D:* The interstitium thins, and the capillaries fuse into a single layer. *Modified from Burri PH. Fetal and postnatal development of the lung. Annu Rev Physiol 46: 617-628, 1984* (22).

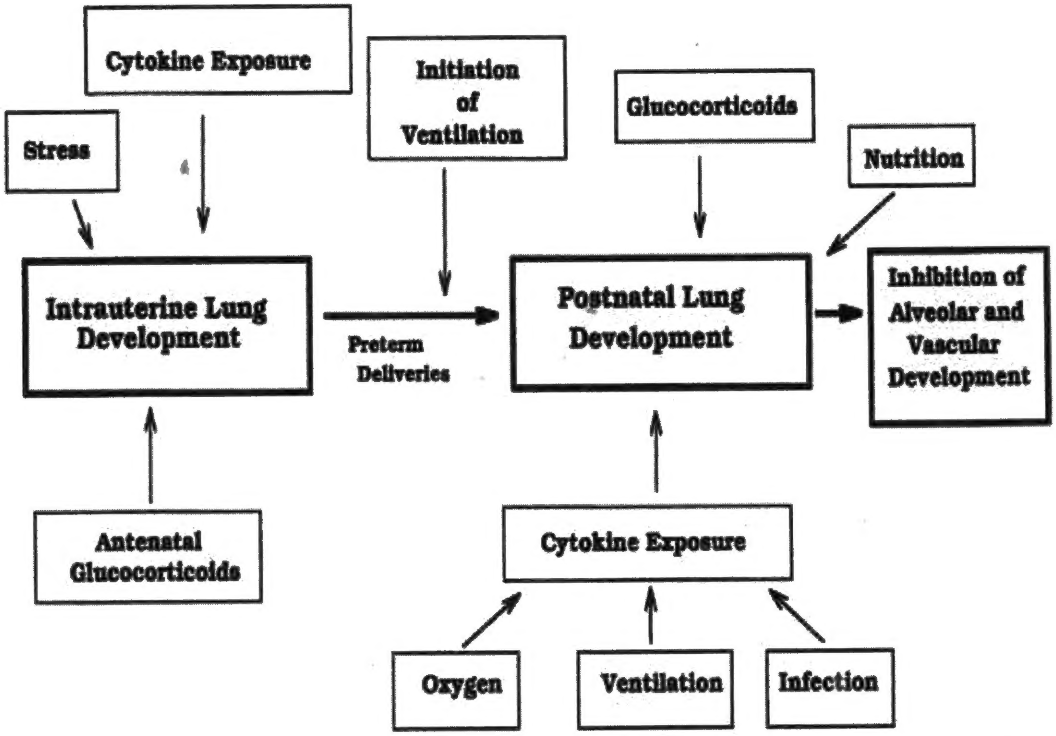
Cytokine Exposure
Stress
Initiation of Ventilation
Glucocorticoids
Nutrition
Intrauterine Lung Development
Preterm Deliveries
Postnatal Lung Development
Inhibition of Alveolar and Vascular Development
Antenatal Glucocorticoids
Cytokine Exposure
Oxygen
Ventilation
Infection

Figure 1.4. Flow-chart of the factors involved in BPD.

This figure describes the factors, such as cytokine exposure and glucocorticoids, which can cause lung damage and BPD, both during gestation and after birth. *Modified from Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res 46: 641-643, 1999 (78).*

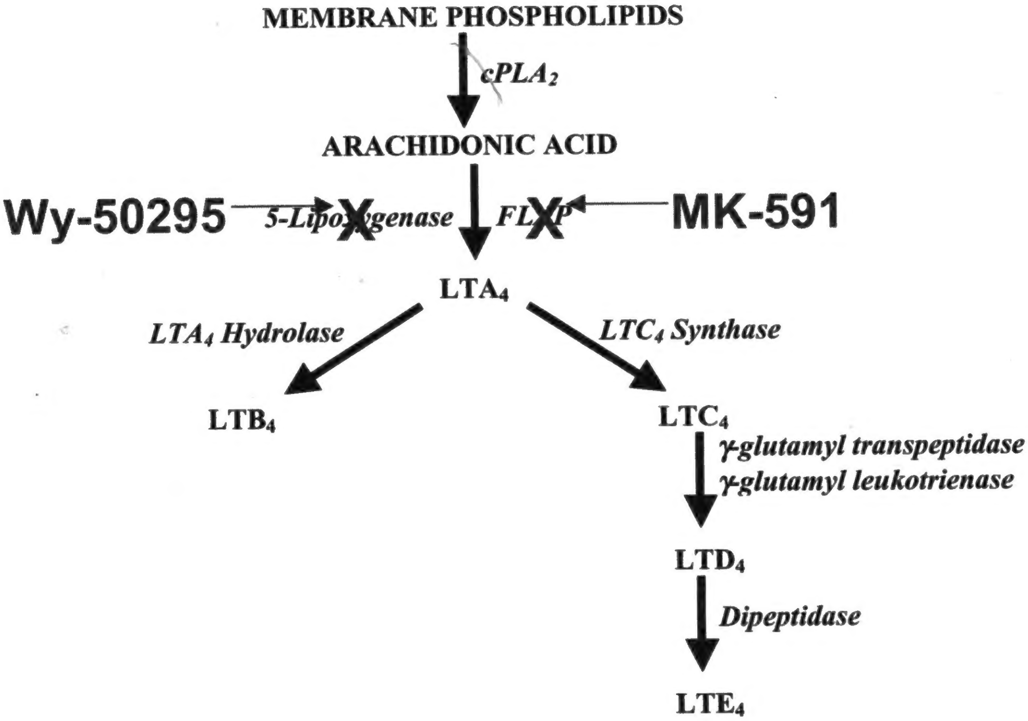

Figure 1.5. Leukotriene synthesis pathway.

This figure illustrates the pathway of leukotriene synthesis. It indicates the major products of the reactions, along with the actions of the LTs. It also indicates LT antagonists Wy-50295 and MK-0591, and the agents that they block, 5-LO and FLAP, as denoted by the large Xs.

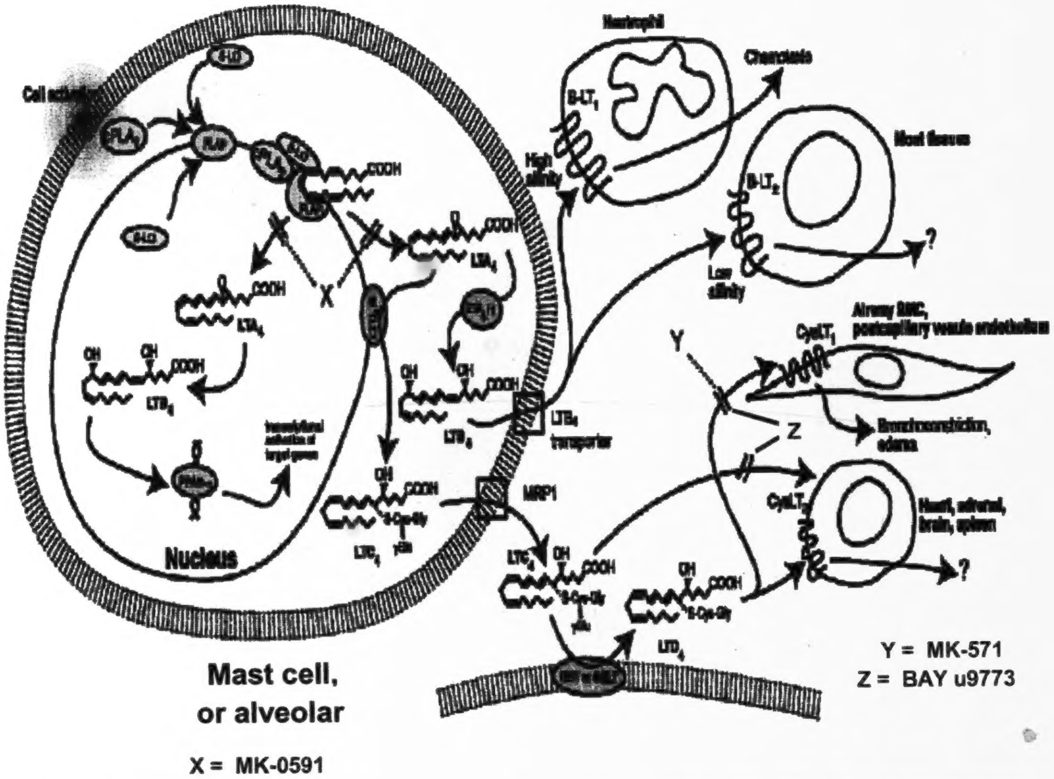

Figure 1.6. LT synthesis pathway and their actions.

This figure demonstrates the LT synthesis pathway. This includes the factors that are involved, and where the LTs exert their actions. Also, it demonstrates where LT synthesis inhibitors and receptor antagonists act. *Modified from Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294: 1871-1875, 2001 (48).*

CHAPTER 2. RATIONALE

Leukotrienes are derived from AA and are mediators in pulmonary hypersensitivity reactions. Their presence in asthma has been well documented as they cause bronchial constriction, mucus secretion, and edema (124). Chronic lung disease, or BPD (2), is a condition that is prevalent in preterm infants who are born weighing <1000 g (38) and are then intubated and ventilated. This causes lung damage through high oxygen pressures and barotrauma (44). Our laboratory has:

a. Used a hyperoxic (>95% O_2) model in newborn rats which inhibits alveolarization (the final stage of lung development), and therefore replicates certain aspects of BPD (19);
b. Determined that elevated levels of both LTB_4 (62) and the CysLTs (16, 21) are associated with the inhibition of alveolarization;
c. And finally, the administration of the inhibitors of 5-LO (Wy-50295) and FLAP (MK-0591) restored alveolarization to near normal (16, 98), suggesting that LTs are mediators of the hyperoxic induced arrest of alveolarization.

The final step required to prove that the LTs are mediators is to administer them exogenously, independently of hyperoxia, to demonstrate that they inhibit alveolarization.

Therefore, the **first specific aim** was to administer exogenous LTs to newborn pups in normoxia to determine if this would replicate the effects of hyperoxia on alveolarization. We **hypothesized** that it would replicate the hyperoxic inhibition of alveolarization. We also examined if a concentration related effect exists.

CysLT antagonists are currently used in the treatment of asthma, as they inhibit the effects of the CysLTs in the lung. Previous studies in our laboratory have demonstrated that the LTs are involved in newborn lung disease, and perhaps these antagonists could be used to treat this newborn condition. The **second specific aim** was to administer the $CysLT_1$ receptor antagonist MK-571 to newborn rat pups in hyperoxia to allow for normal lung alveolarization. We **hypothesized** that MK-571 would prevent the inhibition of alveolarization due to hyperoxia, implying a role for hyperoxic-induced LTD_4.

The **third specific aim** was to perform an ontogeny study of the two CysLT receptors in fetal and newborn rat lungs, in both normoxia and hyperoxia. We therefore **hypothesized** that hyperoxia would increase the amount of both $CysLT_1$ and $CysLT_2$ protein.

For the first study we expected that the exogenous LTs would replicate the effects of hyperoxia. We predicted that oxygen exposure would cause not only an increase in LTs but also an up-regulation of the CysLT receptors. In the absence of hyperoxia there may not be as many receptors, and therefore the inhibition of septation may not be as great.

The significance of these studies will be an understanding of the role of leukotrienes in normal lung development and hyperoxia-inhibited alveolarization. Ultimately the goal will be to compare rat lung development with human lung development, and their responses to stresses that disrupt development and lead to disease. It is hoped that these studies will lead to the identification of therapeutic targets.

CHAPTER 3. MATERIALS AND METHODS

3.1 STUDY 1. ADMINISTRATION OF EXOGENOUS LEUKOTRIENES TO NEWBORN RAT PUPS IN NORMOXIA

3.1.1 Animals

Sprague-Dawley albino rats (Charles River Breeding Laboratories, St. Constant, PQ) of both sexes were used in all studies. Pregnant dams were delivered on day 15 of gestation to the Health Sciences Laboratory Animal Service Department of the University of Alberta, and housed in a specific pathogen free suite. All experimental procedures followed the guidelines of the Canadian Council of Animal Care. The dams were given food pellets and H_2O *ad libitum* and maintained on a 12 h light/dark cycle. Within an hour of delivery, all of the pups were randomized into litters of 10. The dams were rotated daily between the litters to minimize variation due to changes in milk or feeding patterns. Newborn rats were used for various reasons: they are inexpensive to house and maintain; have a short gestational period and lifespan; and a short period of alveolarization.

3.1.2 Injections

From d4-13 PN pups of both sexes were administered a subcutaneous (s.c.) injection once daily of 10 μg/kg of exogenous LTs: LTB_4, LTC_4 or LTD_4 (Cayman Chemical, Ann Arbor, MI, USA), vehicle (phosphate buffered saline (PBS)), no treatment but handling, and no handling at all. All animals were weighed daily to determine the volume of vehicle to be administered (5 μl/2 g pup). The drugs were prepared daily by evaporating the ethanol vehicle in which the exogenous LTs were dissolved, and redissolving them in PBS (100 μg/ml). All injections were administered using 30½ G precision glide needles and 1 ml syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

3.1.3 Lung Perfusions

On d14 PN the pups were euthanized with an intraperitoneal injection of 150 μl of pentobarbital sodium (Euthanyl, Bimeda-Mtc Animal Health Inc., Cambridge, ON). A midline laparotomy was performed in the upper abdomen and the internal organs were moved to reveal the diaphragm, which was punctured to create a bilateral pneumothorax. An incision was made in the throat to expose the trachea, two sutures were placed underneath it, and a slit was made on its ventral side. A small catheter was inserted and tied to allow 4% paraformaldehyde to perfuse the tissue for 5 min at 20 cm H_2O (29). After 5 min the catheter was removed and the trachea was tied off with the remaining suture.

In the first set of tissues the rib cage was opened and the lungs were inflated with an open chest. These tissues were not used, however, because the protocol was changed to perfuse with a closed chest. This was done to ensure that the lungs would inflate to a natural state and not hyperinflate. The rib cage was carefully opened so as not to puncture the lung tissue and the lungs were removed.

3.1.4 Tissue Fixation

Once the lungs were removed they were left in 4% paraformaldehyde overnight at 4°C. On day two all excess tissue and debris was removed from the lungs. The bronchi were tied with sutures to separate the right and left lobes. The lobes were weighed in 4%

paraformaldehyde to determine the lung volumes (128). A beaker of 4% paraformaldehyde was placed on a balance and tared. The lobes were lowered into the solution, and the displacement and change in weight was used as a measurement of lung volume for each lobe, and these values were added to determine the total lung volume.

The tissues from the open chest perfusion were weighed transverse sections were cut from the apical, middle and basal portions of the right lobe, and apical and basal portions of the left lobe. In the closed chest tissues, the right lung was separated into its four lobes. All of these tissues were placed in cassettes, and washed 3-times in PBS for 30 min, and left overnight at 4ºC in 0.1 M glycine in PBS. The following day the tissues were dehydrated through a series of 1 h washes, first with 70% ethanol (EtOH), followed by 80% EtOH, 90% EtOH, 95% EtOH, 3-times 100% EtOH, xylene, and left overnight in paraffin wax at 57 ºC. On the last day the tissues were embedded in cassettes for slicing using the microtome (Leica RM2135).

3.1.5 Slide Preparation

The right caudal lobe was embedded with the tip against the bottom of the mold, and the bottom of the tissue at the top of the wax (29). This allows the lung to be sliced into equally spaced sections, which ensures that the results represent the lobe itself and the entire lung. All sections were microtomed at 5 µm, and the first section was taken at 1.6 mm into the lobes. The next nine sections were taken 800 µm apart, for a total of ten sections. These were mounted on slides (Fisherbrand Superfrost Plus, Fisher Scientific, Pittsburg, PA, USA), dried overnight in a 37 °C incubator and were ready for staining with hematoxylin and eosin.

The paraffin wax was removed in two 5 min xylene washes. The tissue was rehydrated in sequential 5 min washes of 100% EtOH twice, 95% EtOH, 90% EtOH, 70% EtOH, 50% EtOH, and ddH_2O. They were stained in Harris Hematoxylin solution (Sigma Diagnostic, St Louis, MO, USA) for 1 min, rinsed in warm running H_2O for 5 min, stained in Eosin Yellowish solution (Fisher Diagnostics, Fair Lawn, NJ, USA) for 3 min, and once again rinsed for 5 min. The slides were dehydrated in sequential 5 min washes of 70% EtOH, 95% EtOH, 100% EtOH twice, xylene waste, and xylene twice. The coverslips (Fisherbrand Microscope Cover Glass, Pittsburg, PA, USA) were mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA), and the slides dried overnight.

3.1.6 Morphometric Analysis

Slides were first randomized, and coded to ensure that the analysis was blinded. The first, second, and tenth sections were not included in the analysis. The remaining seven sections were analyzed for each tissue, and 6-8 tissues were used per treatment group. The large right lobe was used because previous studies have demonstrated that any lobe of the lung can be used, since no differences have been found between them (151). Morphometric analysis was used to determine the volume densities of the parenchymal and non-parenchymal tissue using the point count analysis method (151). This technique was performed using a light microscope (Olympus BX40) with both a motorized stage (Prior Optiscan) and a scope (Olympus U-DA) attached to it, and Image Pro Plus Software (version 4.5.1.23). A point target was superimposed on the section on the stage via the scope. The software was used to identify the left, top, right and bottom boundaries of the tissue, which established a grid pattern. The motorized stage then traveled sequentially frame by frame following the grid. Each parameter that the target falls in each frame was counted using the 20x objective. Parameters measured were airspace, septa, pleura, bronchi lumen and walls, and vessel lumen and walls.

3.1.7 Effect of Exogenous Leukotrienes on Organ Weights

Injections of vehicle (n=5), No Tx (n=2), LTB_4 (n=4), LTC_4 (n=4), and LTD_4 (n=5) in the same manner as previously described. The pups were euthanized and the lung perfusion technique was the same as that previously described. However, once the pneumothorax was created, the rib cage was carefully opened to not puncture the lung tissue. Tweezers were used to remove the right lobe of the lung by pinching the hilus and carefully pulling the tissue away from the heart. The lungs were washed in PBS to remove any debris or blood from their surface, weighed, frozen in liquid nitrogen, and were stored at –80°C. The liver, heart, right kidney, spleen and thymus were removed in the same manner.

3.1.8 Protein Extractions

Protein was extracted from all lung samples collected in the organ weight study. The tissue was ground in liquid nitrogen, in a frozen mortar using a pestle, and weighed, with the majority of the samples weighing between 0.1-0.2 g. Lysis buffer (1 ml) (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS with phosphatase and protease inhibitors) was added and the tissues were homogenized using a microprobe 3-times for 10 s. The samples were centrifuged 2-times at 10,000 X gravity for 10 min in the cold room. The supernatant was collected, aliquoted and stored at –80°C.

3.1.9 BCA Assay

Samples of the supernatant were used to determine the concentration of protein using a micro BCA assay protein reagent kit (Pierce, Rockford, IL, USA). The samples were diluted in lysis buffer to 1/30, 1/75 and 1/150 in triplicate on a 96 well plates, and all readings obtained were within the limits of the standard curve (0-30 μg of bovine serum albumin (BSA)/well). Reagent solution was added to each well and the plates were incubated at 37°C for 2 h. A plate reader (562 nm) determined the optical density of each well, and this value was compared to the standard curve to calculate the amount of protein per well.

3.1.10 Concentration Response of LTC_4 and LTD_4

Animals were treated with exogenous LTs (LTC_4 and LTD_4), as previously described; to determine if there were concentration related differences in alveolarization. The only change in protocol was a difference in concentration: 20 μg/kg, 50 μg/kg, and 100 μg/kg and vehicle. For the LTD_4 concentration response two pups were administered vehicle, two were treated with 20 μg/kg, three with 50 μg/kg, and three with 100 μg/kg. In the LTC_4 concentration response, there were four treatment group (n=5) that were administered the same concentrations of LTs.

3.2 STUDY 2. ADMINISTRATION OF MK-571 TO NEWBORN RAT PUPS IN BOTH NORMOXIA AND HYPEROXIA

3.2.1 Hyperoxic Exposure

At birth the pups were randomized into litters of 13 pups, which were placed in 0.14 m^3 Plexiglass chambers from d4-14 PN in both hyperoxic (>95% O_2) and normoxic (21% O_2 or air) exposure. The O_2 concentrations were monitored daily (Ventronic oxygen analyzer no. 5517, Temecula, CA, USA), and the CO_2 was filtered with barium hydroxide lime (Baralyme; Chemtron Medical Division, St. Louis, MO, USA). The environment was maintained at 26°C and the humidity at 75-80%. The chambers were opened for less than 15 min daily to switch the dams into normoxia, inject and weigh the pups, and clean the cages.

3.2.2 Injections

There were four treatment groups in this study: Air + vehicle (n=7), Air + MK-571 (n=6), O_2 + vehicle (n=7), O_2 + MK-571 (n=6). The pups were injected from d4-13 PN, s.c., once daily with 25 µg/kg of MK-571 (generous gift from Merck-Frosst, Dorval, PQ). The volume of vehicle injected was dependent on the weight of the pup (5 µl/2 g pup). The vehicle for this study was H_2O, and a stock solution of drug was prepared in advance and kept away from sunlight.

3.2.3 Tissue Processing

The lungs were perfused and analyzed in the same manner as was previously described.

3.3 STATISTICAL ANALYSIS

A one-way analysis of variance was performed using SigmaStat software (version 2.0) to compare treatment and control groups. If a significant F value was observed then a post hoc Tukey's test was used to separate treatment effects. Data was considered significant at $p<0.05$, and all data was expressed as mean ± standard error of the mean (SEM). A Student's t-test was used to determine sex differences within the treatment groups. The desired power of the performed tests was 0.800.

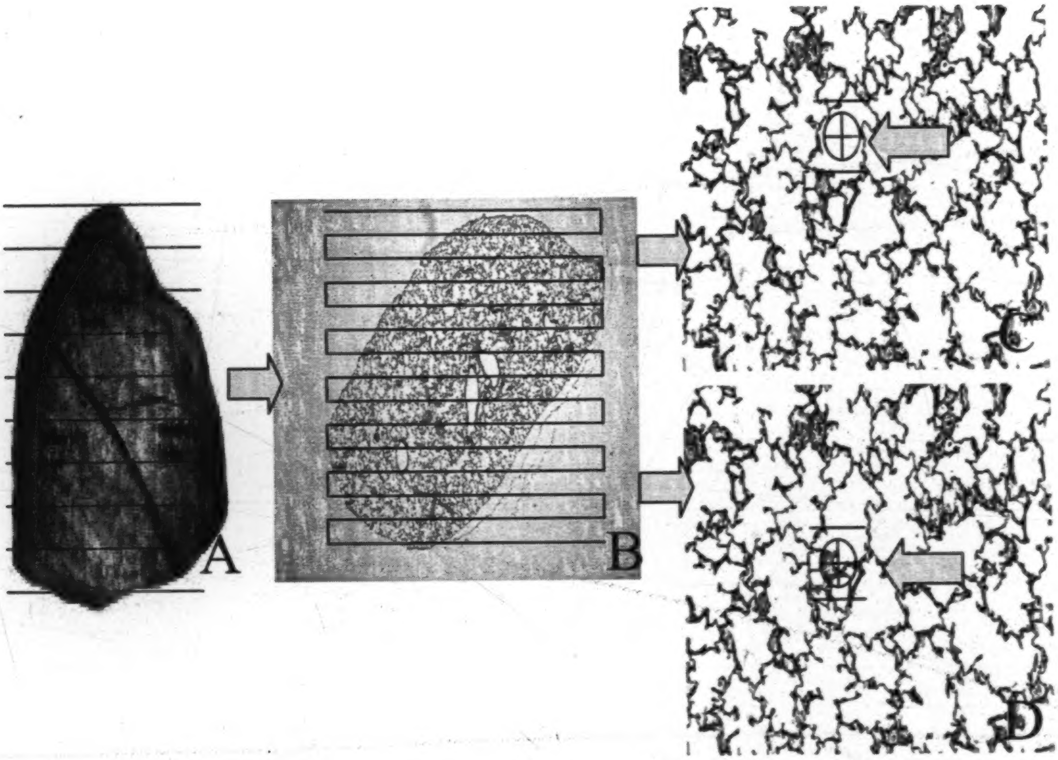

Figure 3.1. Description of the morphometric analysis technique

A: The right lobe is sliced to obtain 10 transverse sections through the lobe. *B*: The edges of a section are identified, and the software travels through the section frame by frame. A point target is overlaid on the tissue using a scope to record the parameters the target hits on each frame. *C*: Target on airspace. *D*: Target on septa. (www.newhopehealthclinic.com/lung_c1.jpg).

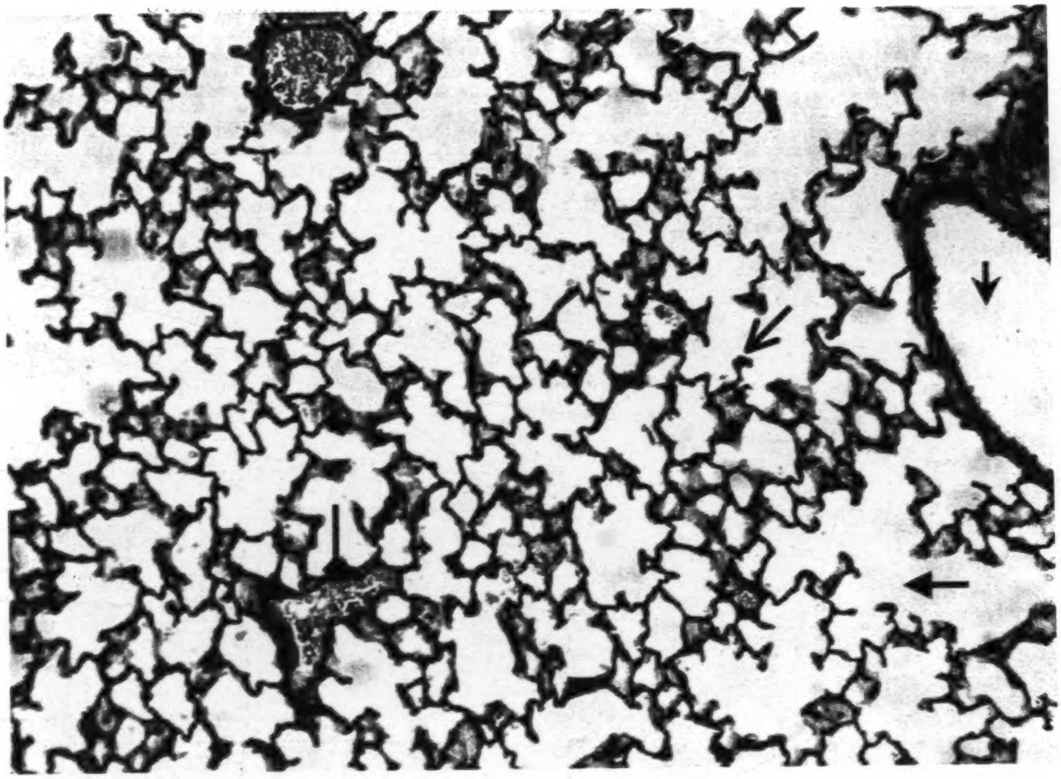

Figure 3.2. Sample parameters measured using morphometric techniques.

Five parameters were measured during morphometric analysis. They were airspace (➔), septa (→), bronchi (↳), vessels (—◆), and pleura (not shown)

CHAPTER 4. RESULTS

4.1 STUDY 1 Administration of Exogenous Leukotrienes to Newborn Rat Pups in Normoxia

4.1.1 Effect of Exogenous Leukotrienes- Open Chest

This study was performed to practice techniques and obtain preliminary data. Therefore, the powers of the test were lower than 0.800 (0.43-0.65). Three groups of newborn rats were treated with 10 µg/kg of LTC_4 (n=9), LTD_4 (n=10) or vehicle (n=10), and weighed daily from d4-13 PN.

There were no changes in the initial and final body weights (Table 4.1). Changes in body weight due to treatment were determined by subtracting the final by the initial body weights (Figure 4.1). There was a significant increase ($p<0.05$) in the LTD_4 group when compared to control. Lung volumes were determined, and there was a significant difference ($p<0.05$) between control and the LTC_4 group (Figure 4.2). The percent body weight of the lungs was calculated by dividing the lung volume (g) by the final body weights (g) of pups and multiplying this by 100 (Figure 4.3). There were significant differences ($p<0.05$) between control and the LTC_4 group. No differences were observed in the treatment groups due to the sex of the pup (data not shown).

Treatment	**Initial Body Weight (g)**	**Final Body Weight (g)**
Vehicle	10.0 ± 0.3	27.8 ± 0.5
LTC_4	10.0 ± 0.2	28.5 ± 0.4
LTD_4	10.1 ± 0.3	29.0 ± 0.5

Table 4.1. Initial and final body weights from the open chest study.

The newborn rat pups were weighed from d4-14 PN. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no statistical differences among the groups for either the initial or final days.

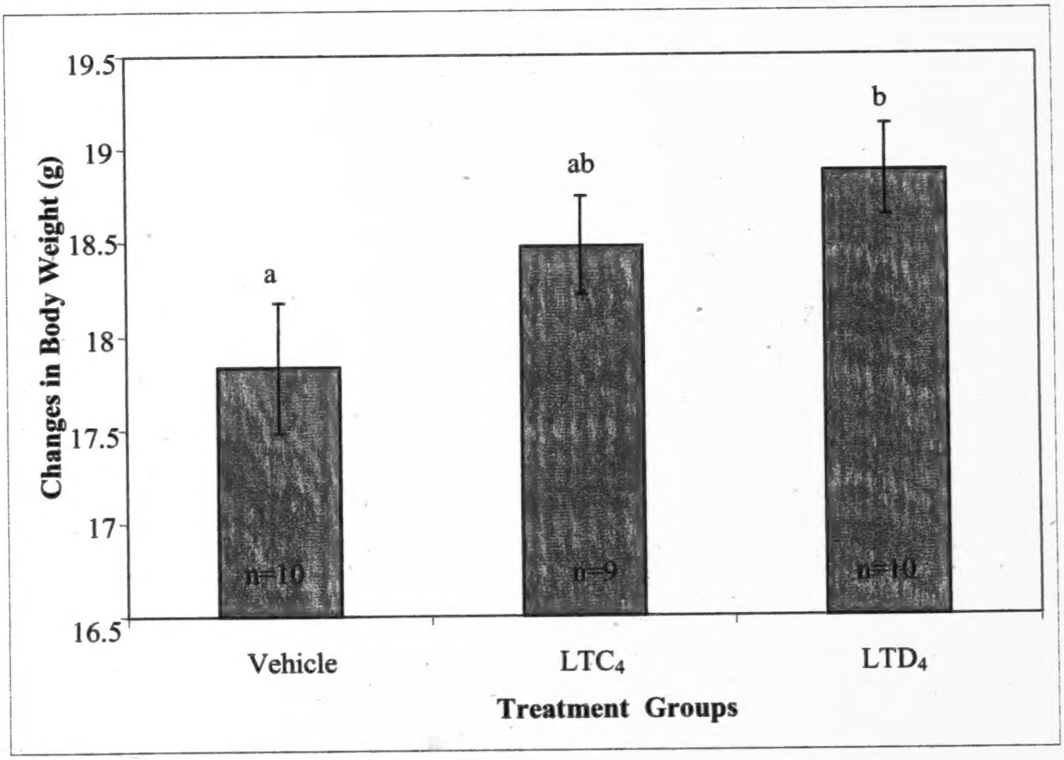

Figure 4.1. Changes in body weight for the open chest treatment groups.

The changes in body weight were determined by subtracting the initial from the final body weight. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase in the LTD_4 group compared to control.

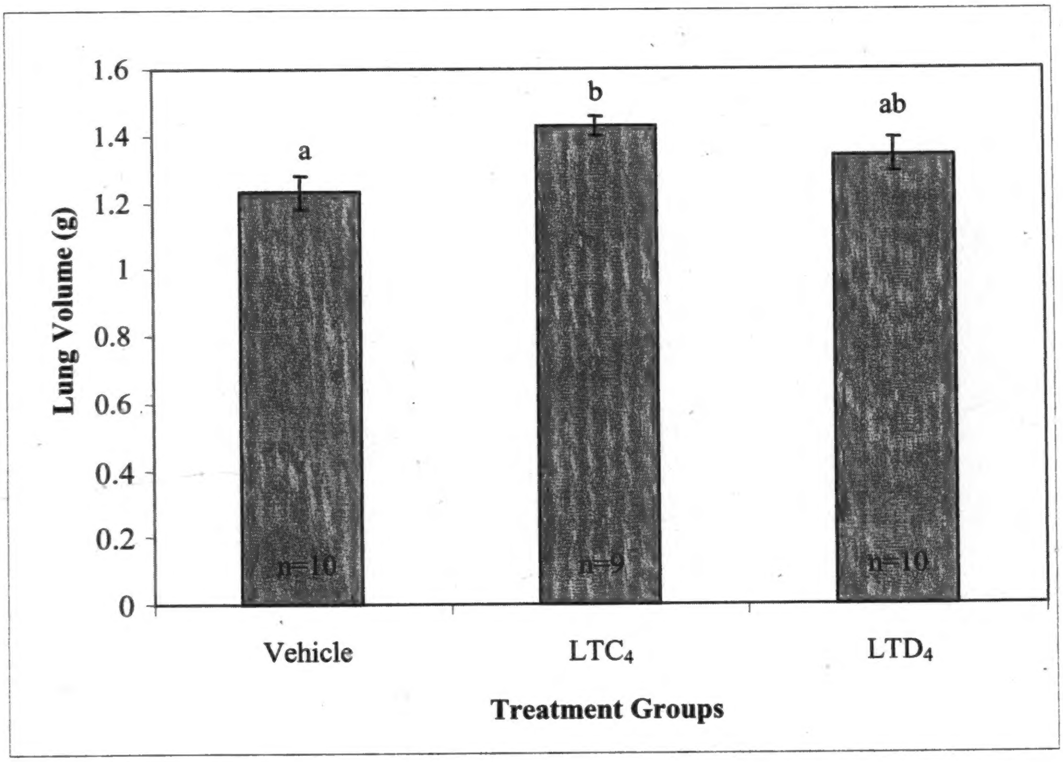

Figure 4.2. Lung volumes for the open chest treatment groups.

The lung volumes were determined by weighing the lungs in 4% paraformaldehyde on a balance. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase in the LTC_4 group as compared to control.

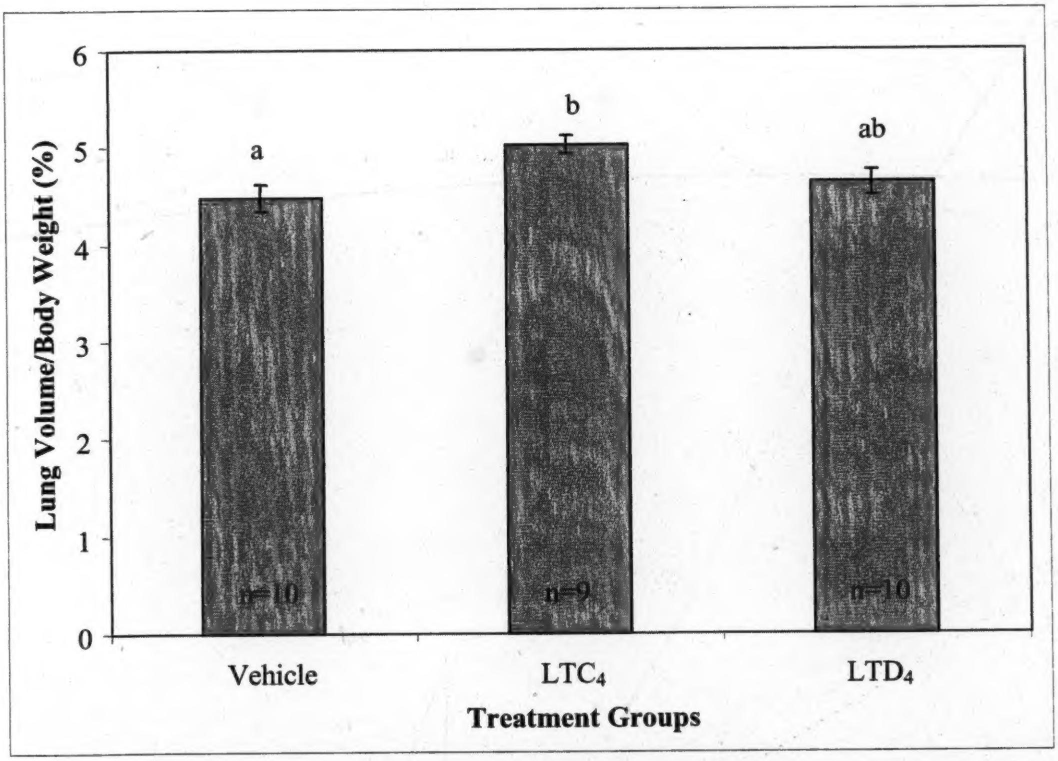

Figure 4.3. The percent body weights of the lungs (g/g BW *100) for the open chest treatment groups.

The percent of the body weight of the lungs was calculated. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. An increase was observed in the LTC_4 group as compared to the vehicle control group.

4.1.2 Effect of Exogenous Leukotrienes on Organ Weights

The newborn rat pups were treated with 10 μg/kg of exogenous LTs, and the five groups were the same as those from the previous study: vehicle (n=5); No Tx (n=2); LTB_4 (n=4); LTC_4 (n=4); and LTD_4 (n=5). There were no differences between the vehicle and No Tx groups, and the data for the No Tx group were not shown. This study was performed as a pilot study to determine the effects of the exogenous LTs on organ weights. Therefore, the powers of the study were lower than 0.800 (0.49-0.795).

There were no differences in the initial and final body weights between the groups (Table 4.2). To determine if treatment had any effect on the changes in body weights, the final body weight was subtracted by the initial body weight (Figure 4.4). There was a significant difference ($p<0.05$) between the LTC_4 and LTD_4 groups. Six organs (right lung, liver, heart, right kidney, spleen and thymus) were removed and weighed (Figure 4.5). There was a significant difference ($p<0.05$) in the liver weights of the LTD_4 group as compared to the other groups. The % body weight of these organs was determined, and again only the livers of the LTD_4 group were significantly different ($p<0.05$) (Figure 4.6).

Treatment	Initial Body Weight (g)	Final Body Weight (g)
Vehicle	12.0 ± 0.8	29.9 ± 1.0
LTB_4	10.2 ± 0.2	29.9 ± 0.8
LTC_4	11.3 ± 0.2	31.4 ± 0.9
LTD_4	11.3 ± 0.4	28.9 ± 0.8

Table 4.2. Initial and final body weights from the organ weight treatment groups.

The newborn rat pups were administered 10 µg/kg of LTs, and weighed from d4-14 PN. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no statistical differences among the groups for the initial and final days.

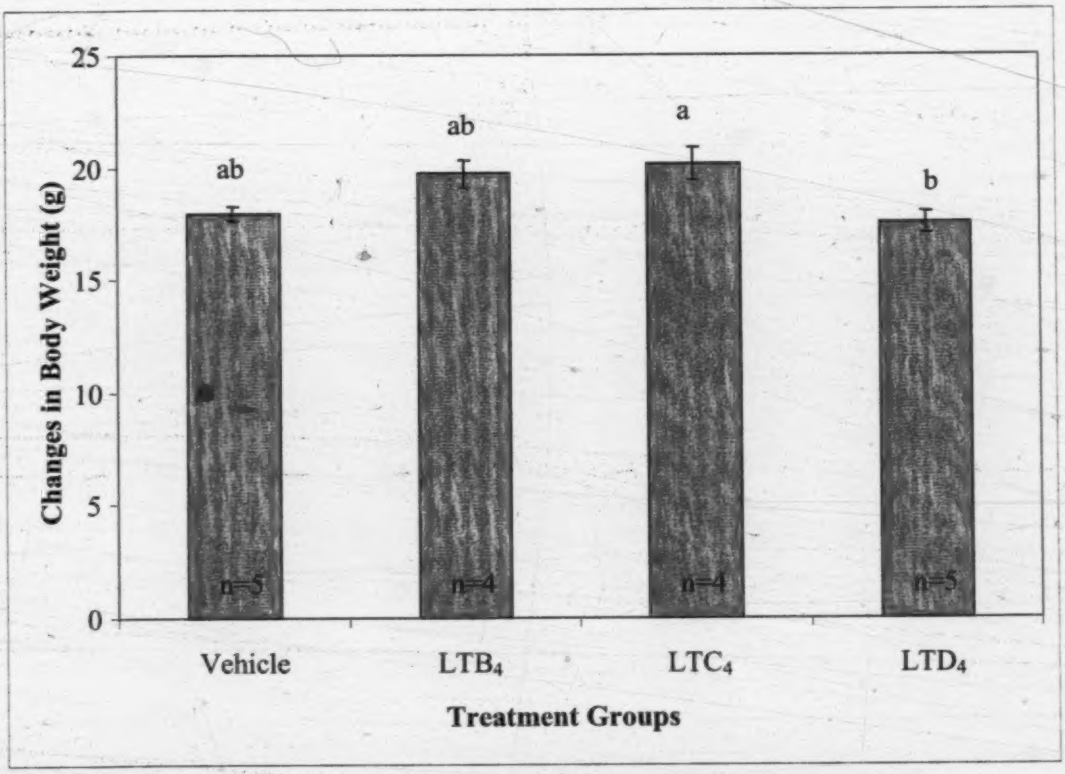

Figure 4.4. Changes in body weight for the organ weight treatment groups.

The changes in body weight were determined by subtracting the initial from the final body weight. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant difference between the LTC_4 and the LTD_4 groups.

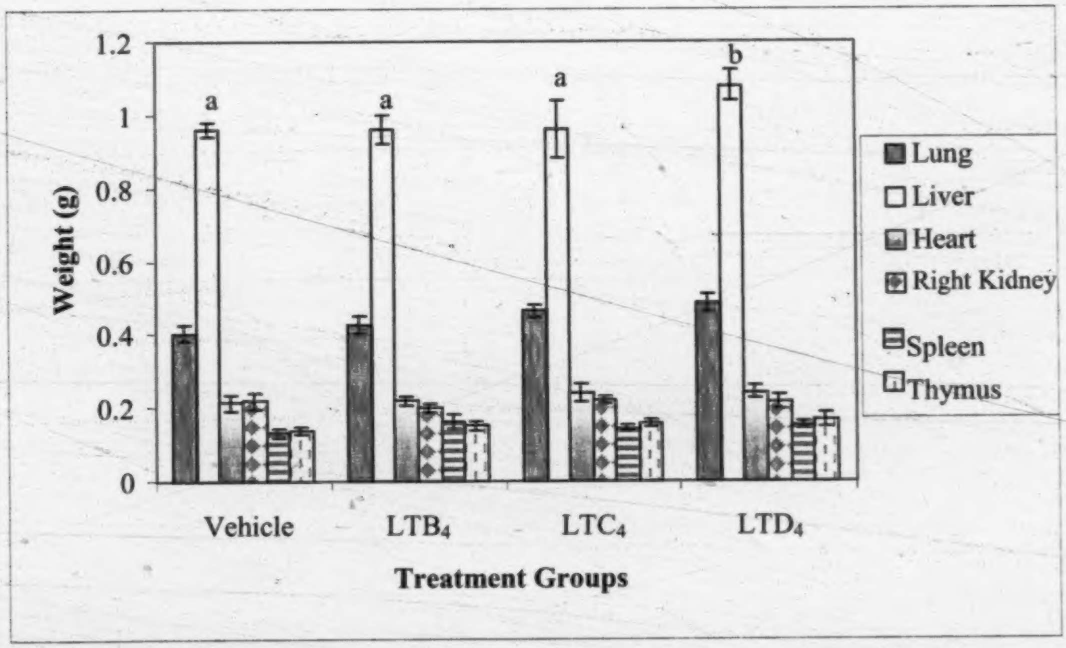

Figure 4.5. Weights of the organs.

The organ weights of the right lung, liver, heart, right kidney, spleen and thymus of the newborn rats. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and there were 4-5 pups per group. There was a significant difference in liver weight in the LTD_4 group as compared to the other three groups.

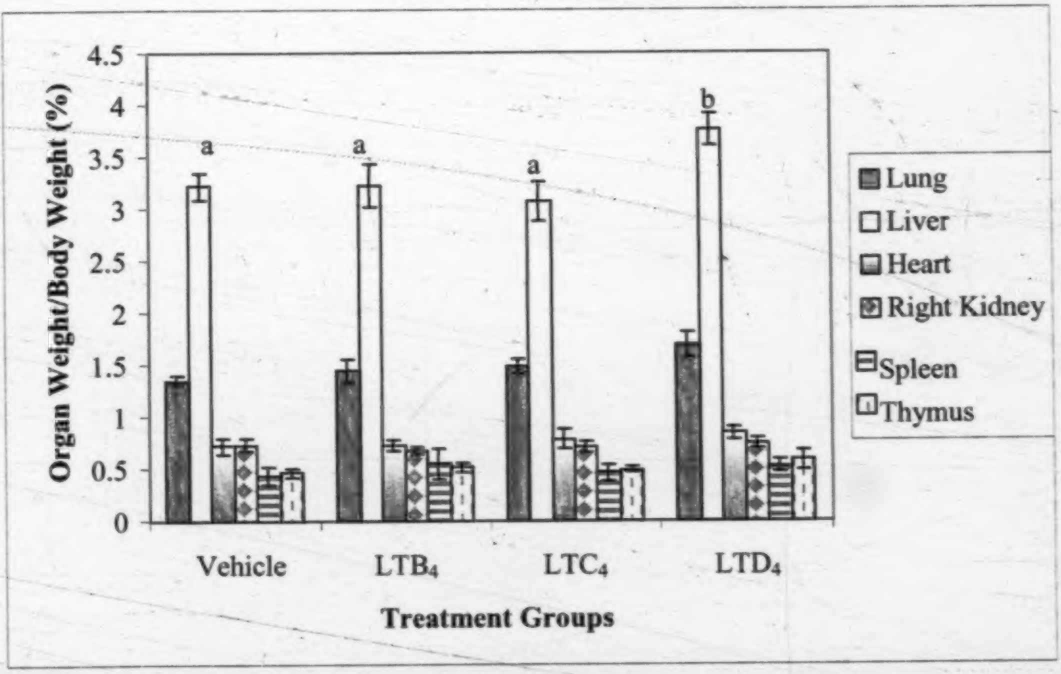

Figure 4.6. Percent body weights of the organs (g/g BW *100) for the organ weight treatment groups.

The percent of the body weight of the organs was calculated. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and there were 4-5 pups per group. There was a significant difference in the % body weight of the livers in the LTD_4 group as compared to the other three groups.

4.1.3 Effect of Exogenous Leukotrienes- Closed Chest

Newborn rat pups were grouped (n=10) and treated from d4-13 PN: 10 µg/kg of LTB_4, LTC_4, LTD_4; vehicle; and a group that was not treated (No Tx) but was weighed and handled daily. A third control group, a group that was not handled until day 14, was also weighed on day 14. The final body weights of the three control groups (vehicle, No Tx, and the no handling group) were compared (Figure 4.7). There were significant differences ($p<0.05$) between all three groups, and the power was significant (1.000). The outcome measures of this study were to determine the effects of exogenous LTs on alveolarization using morphometric analysis. The first set of data (body weights, changes in body weights, lung volumes and % body weight) were measured during the lung perfusions to determine trends; therefore the power of the tests were lower than 0.800 (0.449-0.723).

The initial and final body weights were determined (Table 4.3), and even after randomizations there were significant differences ($p<0.05$) in the initial body weights. This could have caused the differences observed in the final body weights. To examine if the treatments caused changes in body weights, the final body weights were divided by the initial body weights, and multiplied by 100 (Figure 4.8). The LTB_4 group was significantly decreased ($p<0.05$) as compared to both the control and the LTC_4 groups. The lung volumes were measured and there were significant differences ($p<0.05$) between the LTB_4 and LTD_4 groups (Figure 4.9). The % body weight of the lung volume was determined (Figure 4.10), and there was a significant decrease ($p<0.05$) in the LTD_4 group when compared to vehicle control.

Morphometric analysis was performed on the tissues. To ensure that the measurements using the right caudal lobe represented the entire lung, 80-85% of the point targets must land on parenchyma, and 15-20% on non-parenchyma. This was demonstrated in the sample measurement for the vehicle control group (Figure 4.11). Approximately 84% of the targets landed on parenchymal tissues, while 16% landed on non-parenchymal tissues. The morphometric results of the vehicle control and the No Tx groups were compared (Figure 4.12). There were no differences between the two groups in the volume densities of either the airspace or septa, therefore only the vehicle control group was used.

There was a significant decrease ($p<0.05$) in the volume density of airspace in the LTD_4 group when compared to the other groups (Figure 4.13), and the power was sufficient (1.000). The volume density of septa was determined (Figure 4.14), and the power was significant (1.000). There was a significant increase ($p<0.05$) in the LTD_4 group as compared to the other three groups. There was also a significant decrease ($p<0.05$) in the LTB_4 group when compared to control. There were no differences in the volume densities of the bronchi or the pleura (data not shown); however, there were differences in the volume density of vessels between LTB_4 and both vehicle and LTC_4, and the power was sufficient (0.885) (Figure 4.15). Using the 20x objective the morphometric data were demonstrated (Figure 4.16). LTB_4 and LTC_4 demonstrate increased airspaces, while LTD_4 visibly had an increased amount of septation as compared to control.

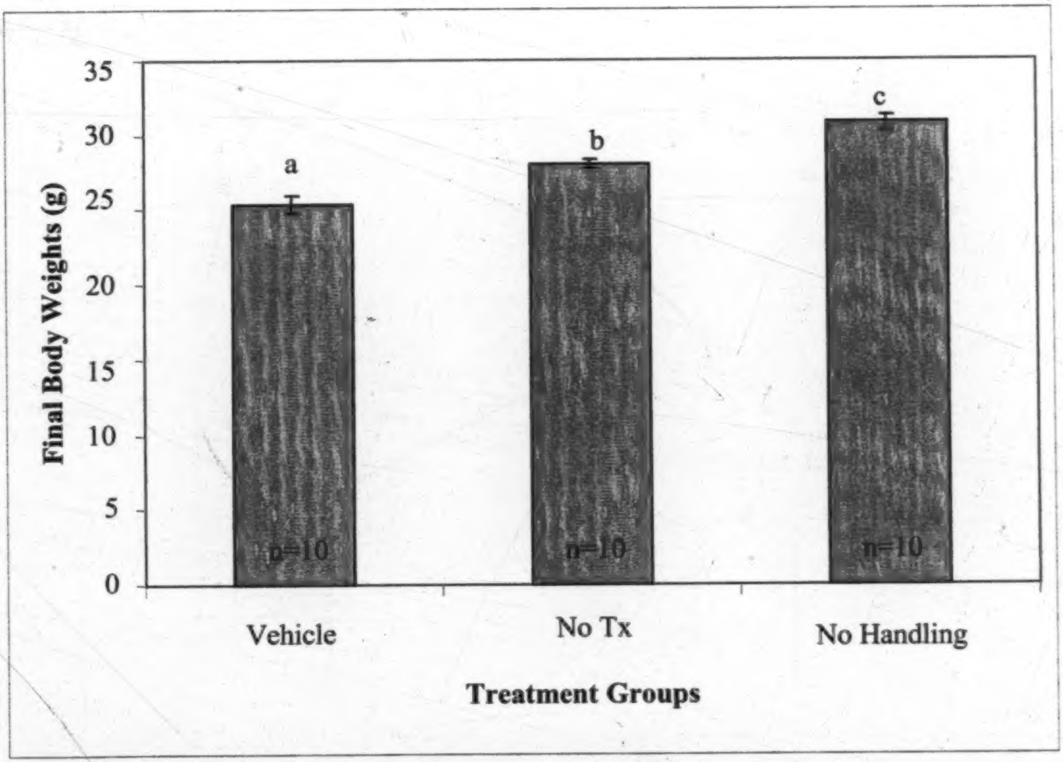

Figure 4.7. Final body weights of three control groups used in the closed chest study.

Comparaison of the final body weights (day 14) between the three control groups: vehicle (PBS), no treatment but being handled and weighed daily, and no handling until day 14. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There were significant differences between the three control groups.

Treatment	Initial Body Weight (g)	Final Body Weight (g)
Vehicle	9.0 ± 0.3^{a}	25.3 ± 0.6^{a}
No Tx	9.3 ± 0.2^{ab}	28.1 ± 0.3^{bc}
LTB_4	11.2 ± 0.4^{c}	29.7 ± 0.7^{c}
LTC_4	9.3 ± 0.2^{ab}	26.2 ± 0.4^{ab}
LTD_4	10.0 ± 0.1^{b}	27.5 ± 0.5^{b}

Table 4.3. Initial and final body weights from the closed chest study.

Newborn rat pups were weighed from d4-14 PN. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$). There were significant differences among the groups for both the initial and the final body weights.

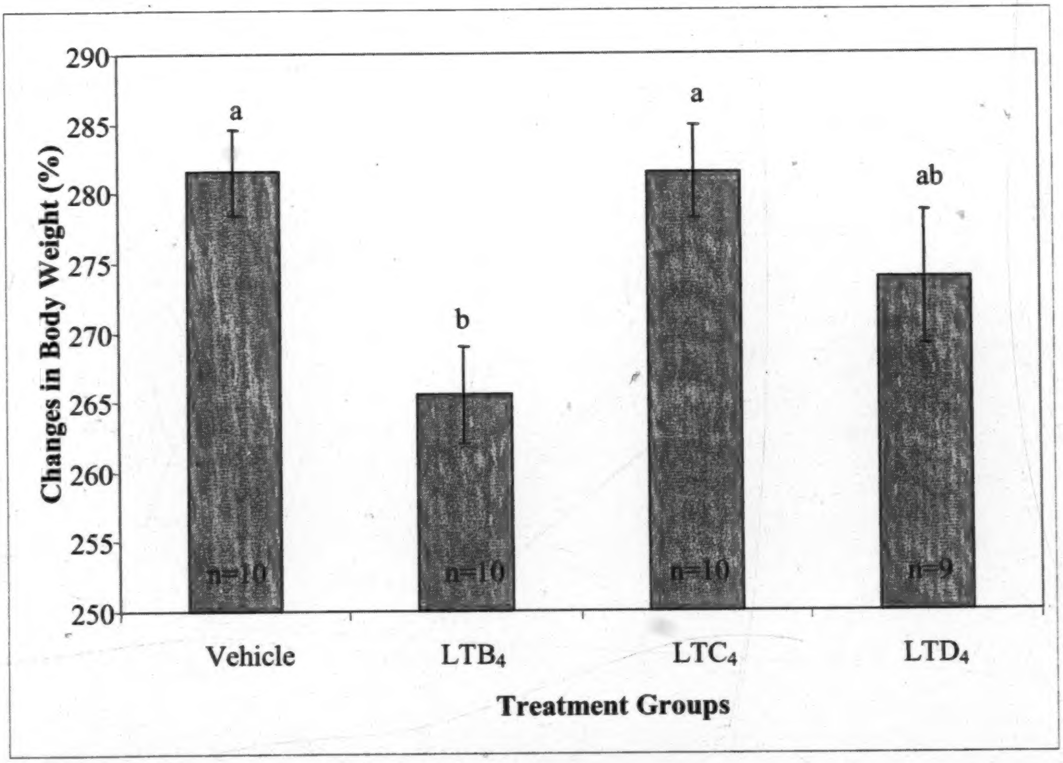

Figure 4.8. Changes in body weight for the tissues treated.

The final body weight was divided by the initial body weight and multiplied by 100. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in the LTB_4 group as compared to both the LTC_4 and the vehicle control groups.

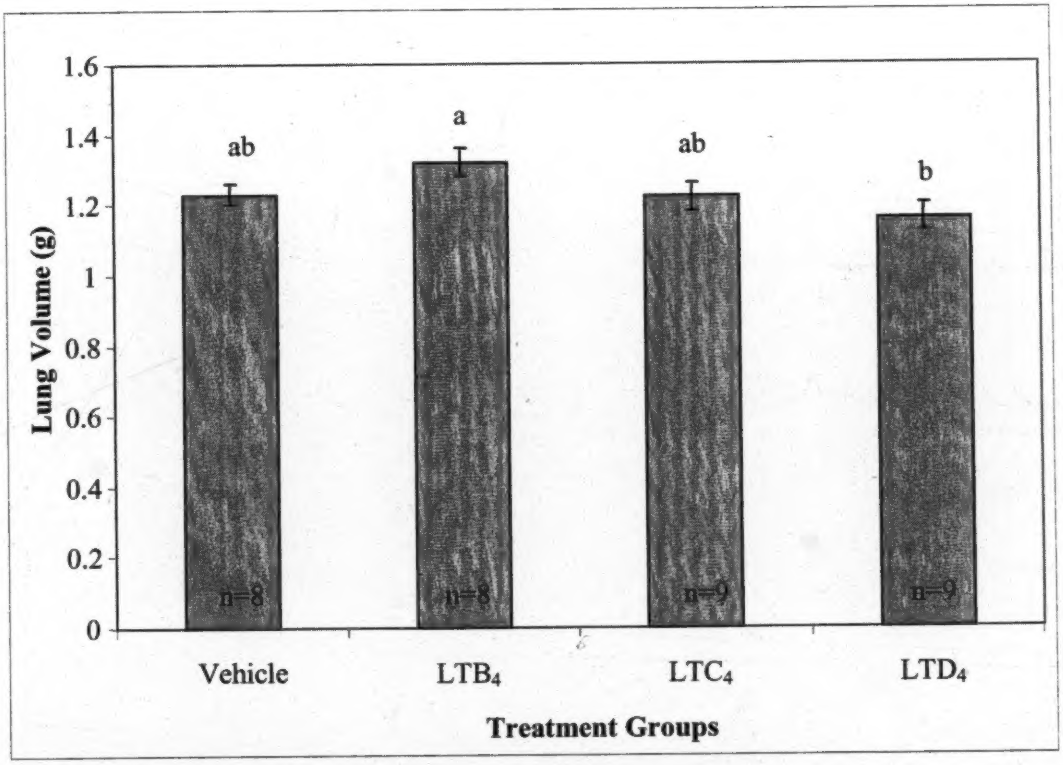

Figure 4.9. Lung volumes for the closed chest treatment groups.

The lung volumes were determined by weighing the lungs in 4% paraformaldehyde on a balance. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant difference between the LTB_4 and LTD_4 groups.

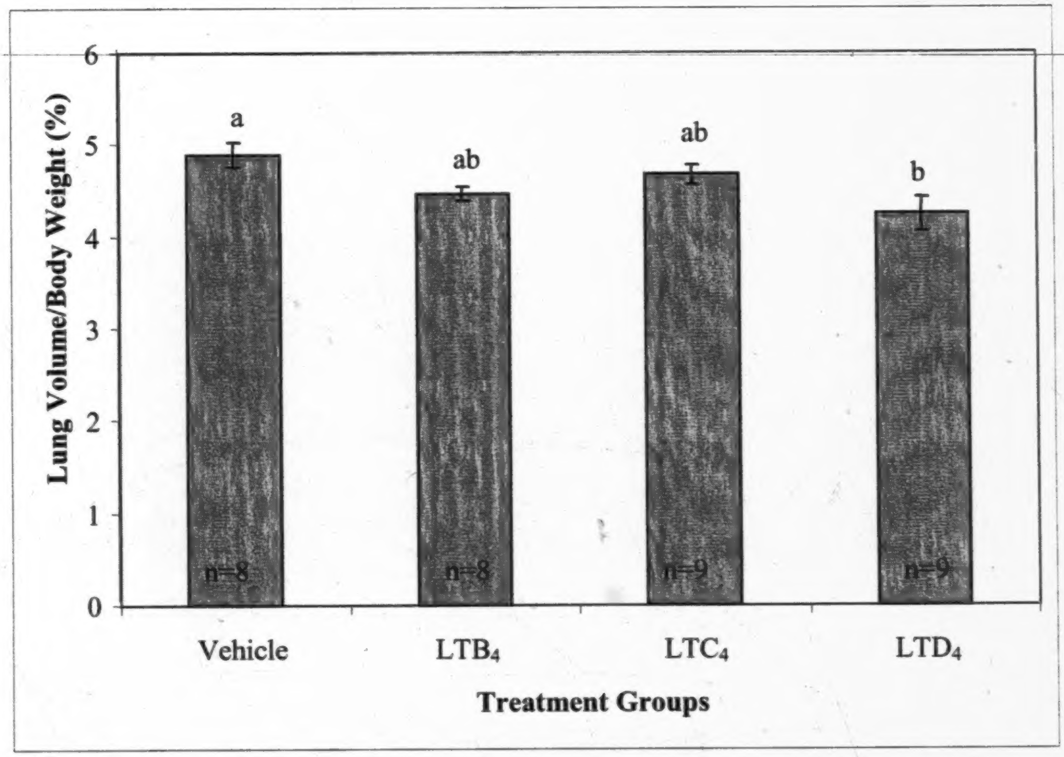

Figure 4.10. Percent body weights of the lungs (g/g BW *100) for the closed chest treatment groups.

The percent of the body weight of the lungs was calculated. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in the LTD_4 group as compared to the vehicle control group.

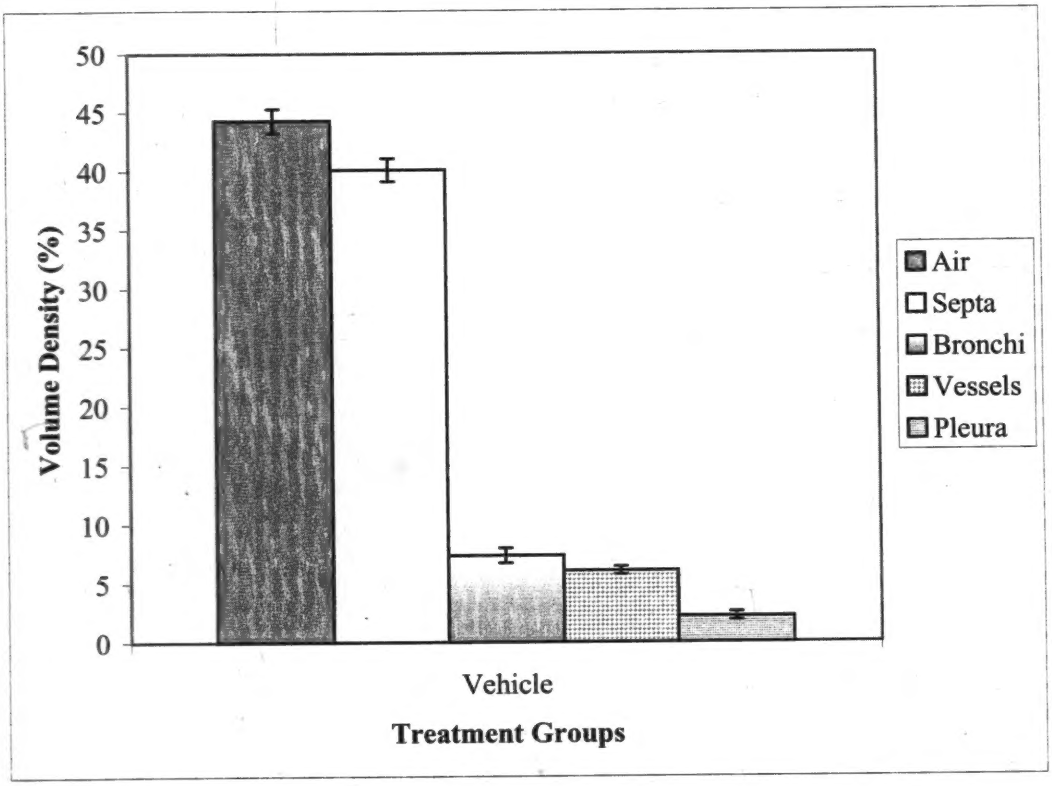

Figure 4.11. Sample measurements of the five parameters for the vehicle control group.

Data from the vehicle control group for the five parameters measured during morphometric analysis. The parenchyma must represent 80-85% and the non-parenchyma 15-20% in one lung lobe to accurately represent the entire lung. These results demonstrate that this occurred using our analysis technique.

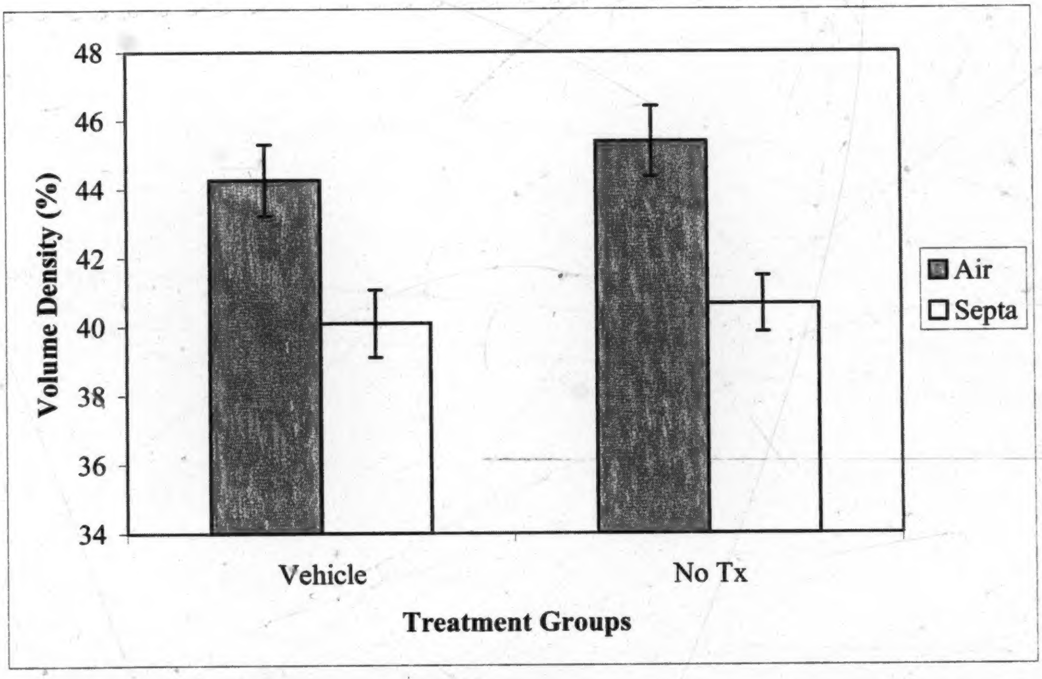

Figure 4.12. Volume densities for the control groups.

Volume densities of airspace and septa in the vehicle and the no treatment control groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. This graph demonstrates that there were no differences between the two groups.

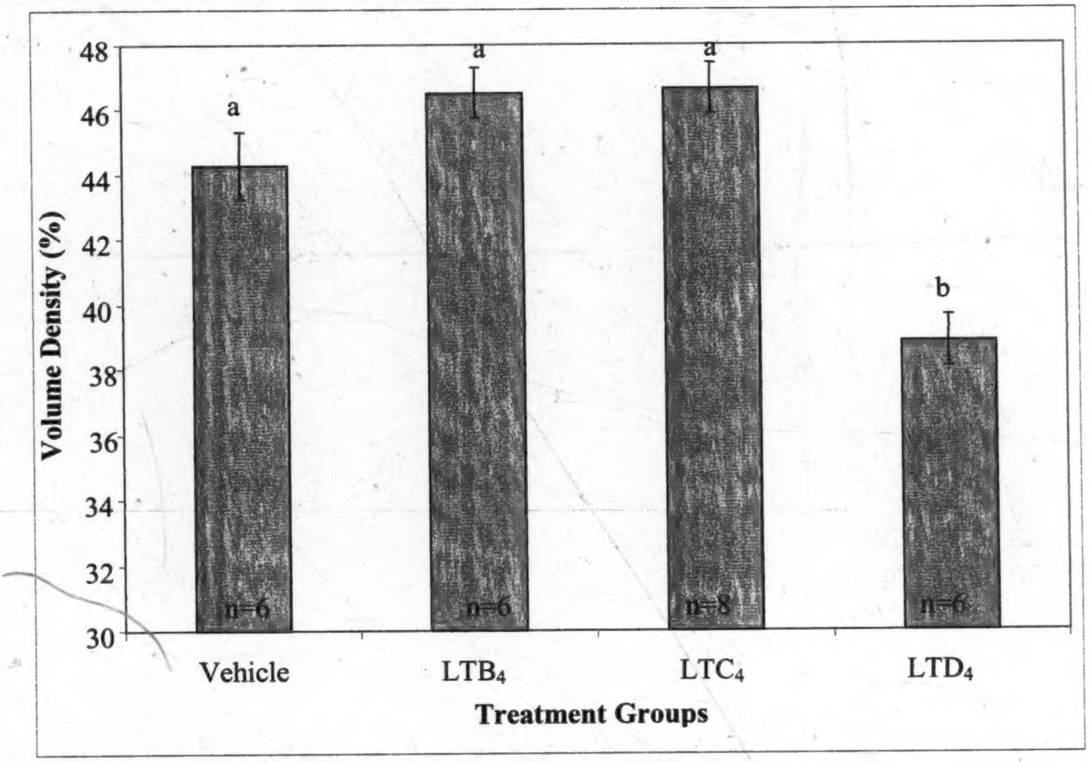

Figure 4.13. Volume density of airspace for the closed chest treatment groups.

The measured volume density of the airspaces of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in the LTD_4 group as compared to the other three groups.

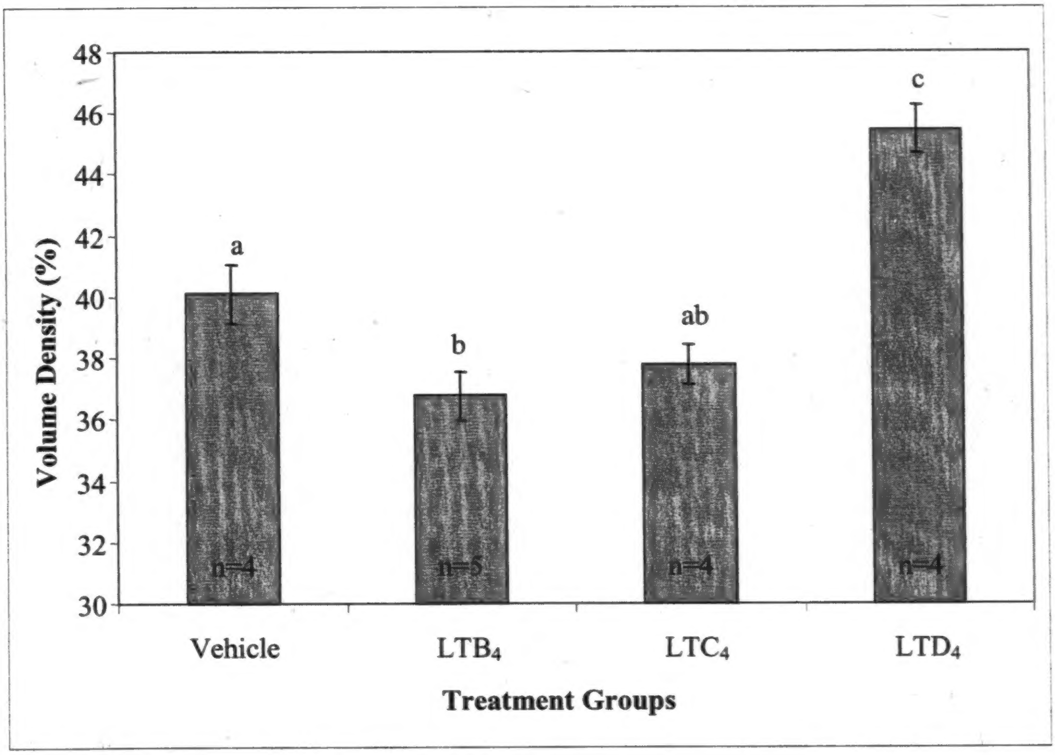

Figure 4.14. Volume density of septa for the closed chest treatment groups.

The measured volume density of the septa of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase in the LTD_4 group as compared to the other treatment groups. There was also a significant decrease in the LTB_4 group as compared to control.

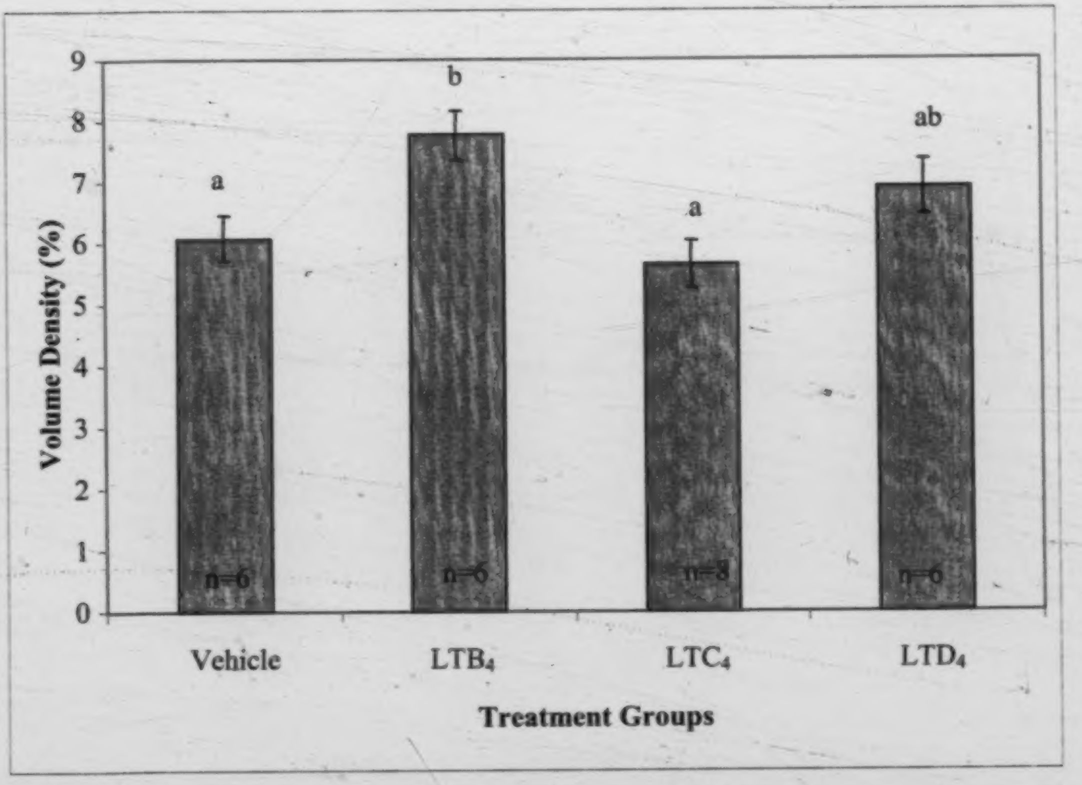

Figure 4.15. Volume density of vessels for the closed chest treatment groups.

The measured volume density of the vessels of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase in the LTB_4 group compared to vehicle control.

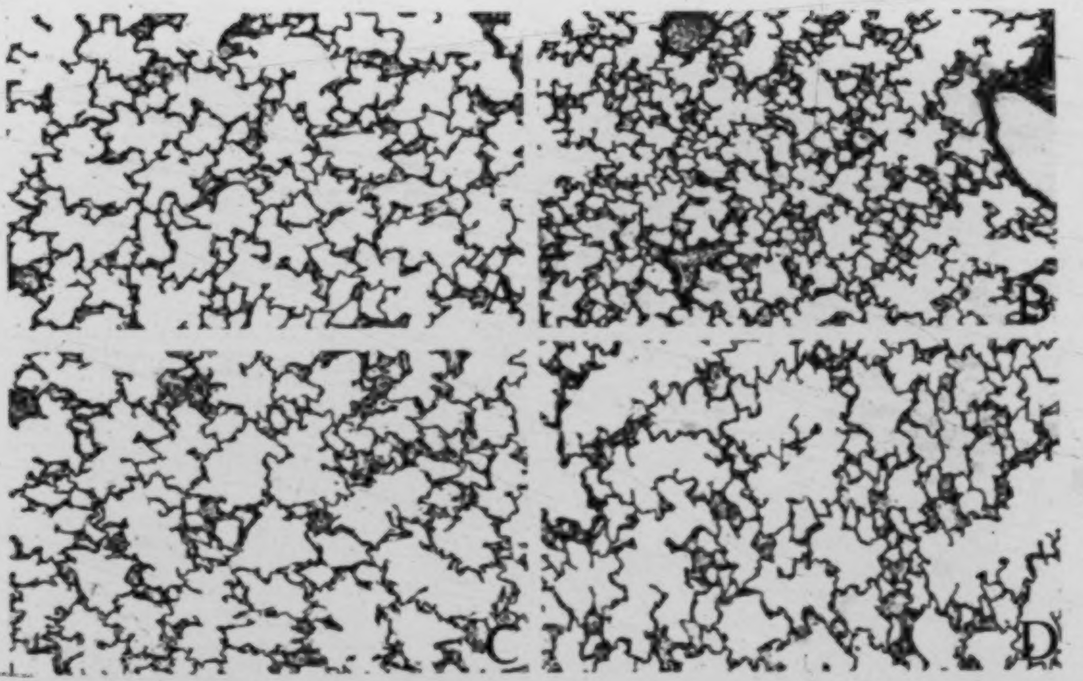

Figure 4.16. Micrographs of the closed chest treatment groups using the 20x objective.

Treatment groups at the 20x objective. *A:* vehicle, *B:* LTD_4; *C:* LTB_4; and D: LTC_4. It was apparent that LTB_4 and LTC_4 have larger airspaces that vehicle control. Also, LTD_4 appears to have increased septation as compared to vehicle control.

4.1.4 Concentration Response of LTC_4

Four groups (n=5) of newborn rat pups were injected for another concentration response study with vehicle, 20 μg/kg, 50 μg/kg, or 100 μg/kg of LTC_4. The pups that were administered the two higher concentrations developed an inflammation at the site of injection on the back of their necks, which became an inflammed, bruised, purple area. There were no differences in both initial and the final body weights of the treatment groups (Table 4.4). There were no changes in body weight among the groups (Figure 4.17). The lung volumes of the treatment groups were calculated, and there were no differences (Figure 4.18). The % body weight of the lungs was also calculated for each group, and again there were no differences between the groups (Figures 4.19).

Morphometric analysis was performed on the tissues to determine which concentration would most effectively inhibit alveolarization. The results for the 10 μg/kg group from the previous study were used to compare it to the concentrations used in this study. There was a significant increase ($p<0.05$) in the volume density of airspace with the 50 μg/kg concentration as compared to vehicle control (Figure 4.20); however, the power was low (0.526). There were significant decreases ($p<0.05$) in the four concentration groups as compared to control (Figure 4.21), and the power was significant (0.952). There were no differences due to treatment in the bronchi, vessels and pleura (data not shown). The micrographs demonstrate that there were larger airspaces in the concentration treatment groups as compared to vehicle control (Figure 4.22), and this corresponds with the morphometric data.

Treatment	Initial Body Weight (g)	Final Body Weight (g)
Vehicle	12.1 ± 0.3	34.2 ± 0.6
20 µg/kg	12.2 ± 0.3	33.4 ± 0.3
50 µg/kg	12.2 ± 0.7	31.3 ± 1.3
100 µg/kg	11.9 ± 0.4	31.6 ± 0.9

Table 4.4. Initial and final body weights from the LTC_4 concentration response.

The newborn rat pups were weighed from d4-14 PN. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no differences among the groups for the initial and final days.

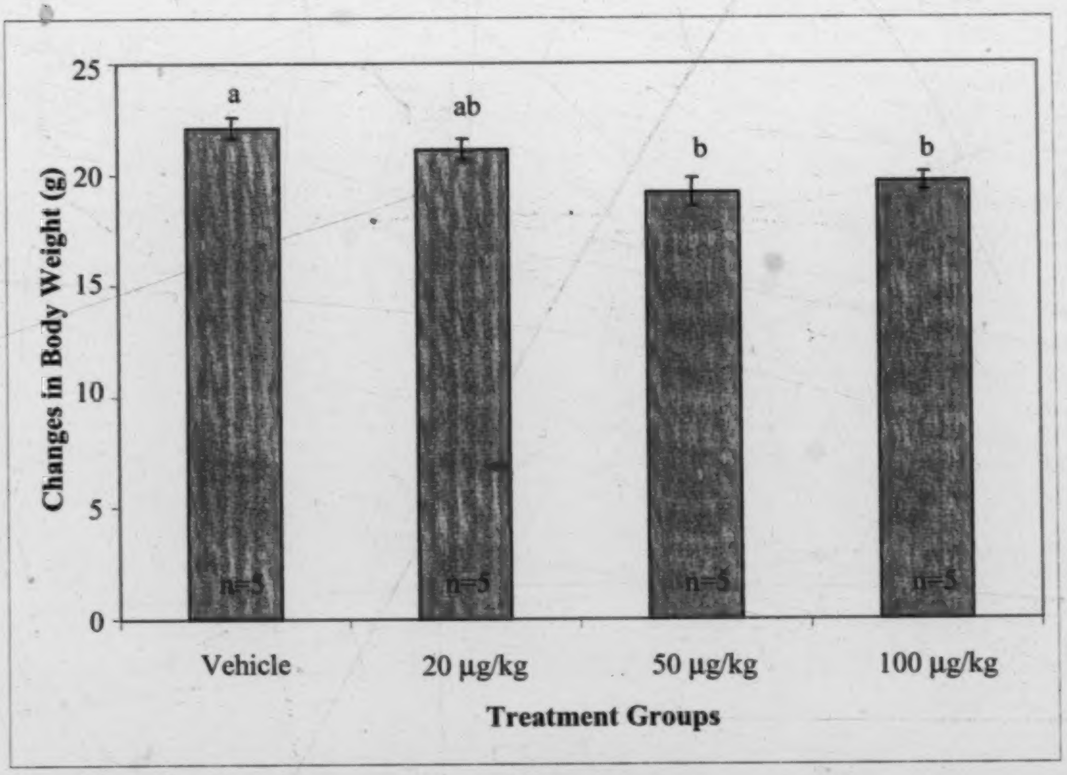

Figure 4.17. Changes in body weight for the LTC_4 concentration response treatment groups.

The changes in body weight were determined by subtracting the initial from the final body weight. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in body weight due to treatment with LTC_4 at 50 μg/kg and 100 μg/kg as compared to vehicle control.

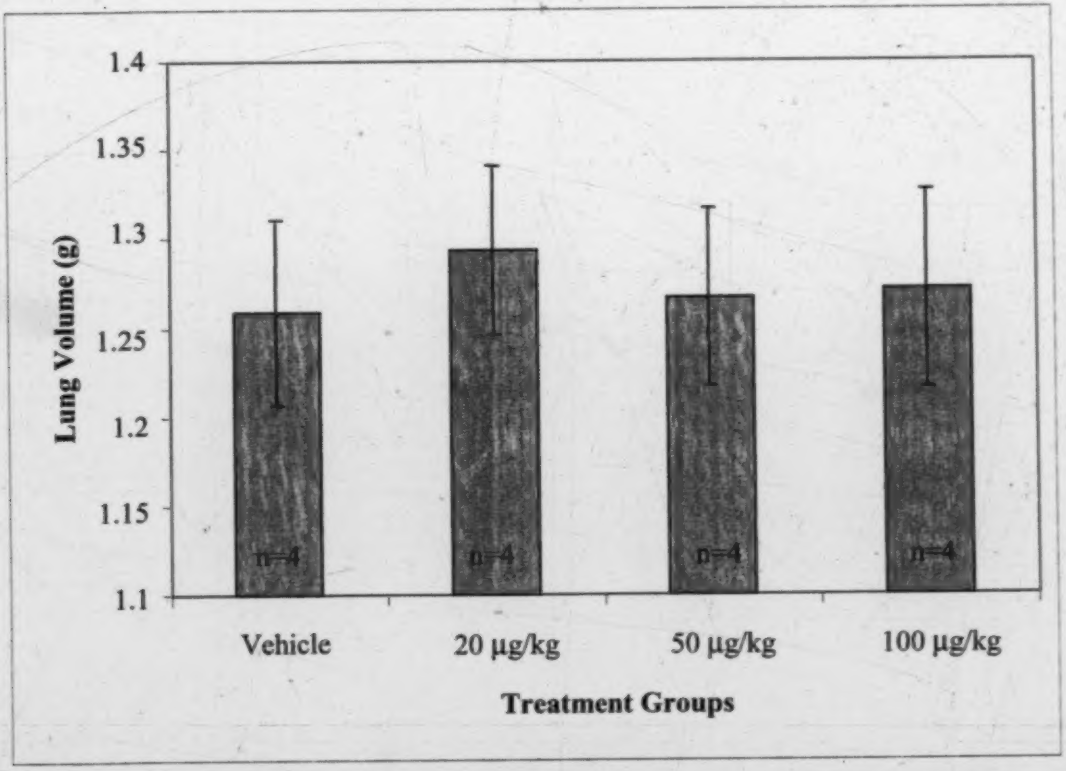

Figure 4.18. Lung volumes of the treatment groups in the LTC$_4$ concentration response.

The lung volumes were determined by weighing the lungs in 4% paraformaldehyde on a balance. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no differences between the treatment groups.

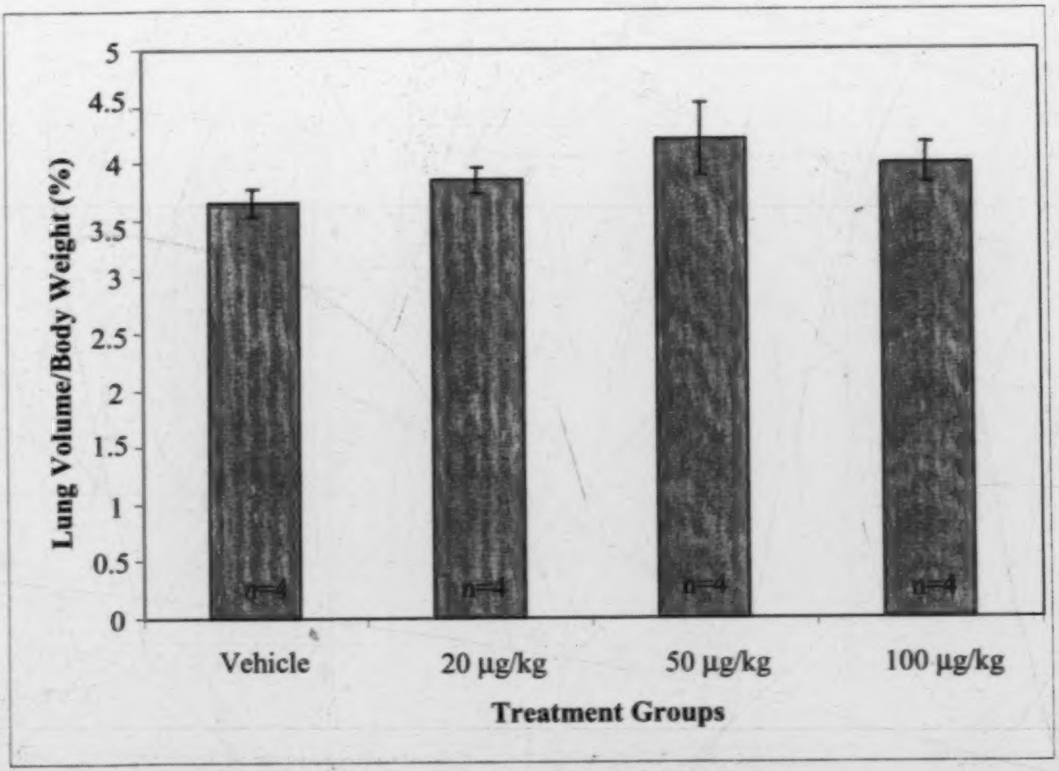

Figure 4.19. **Percent body weights of the lungs (g/g BW *100) for the LTC_4 concentration response.**

The percent of the body weight of the lungs was calculated. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no differences between the groups. These values are smaller than previous studies due to these pups having increased final body weights, although their lung volumes were comparable. These pups were born a day earlier then expected, and we suspect this is the reason for the data in this figure.

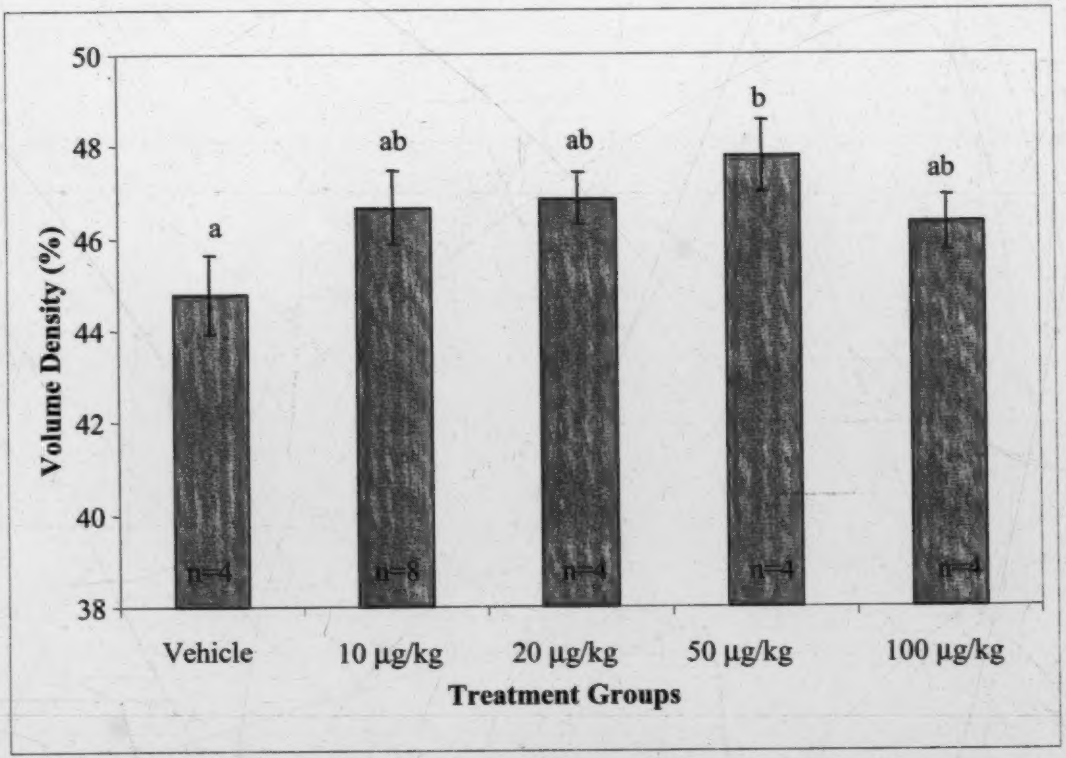

Figure 4.20. Volume density of airspace for the LTC_4 concentration response treatment groups.

The volume density of the airspaces of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase with the 50 µg/kg concentration of LTC_4 as compared to vehicle control.

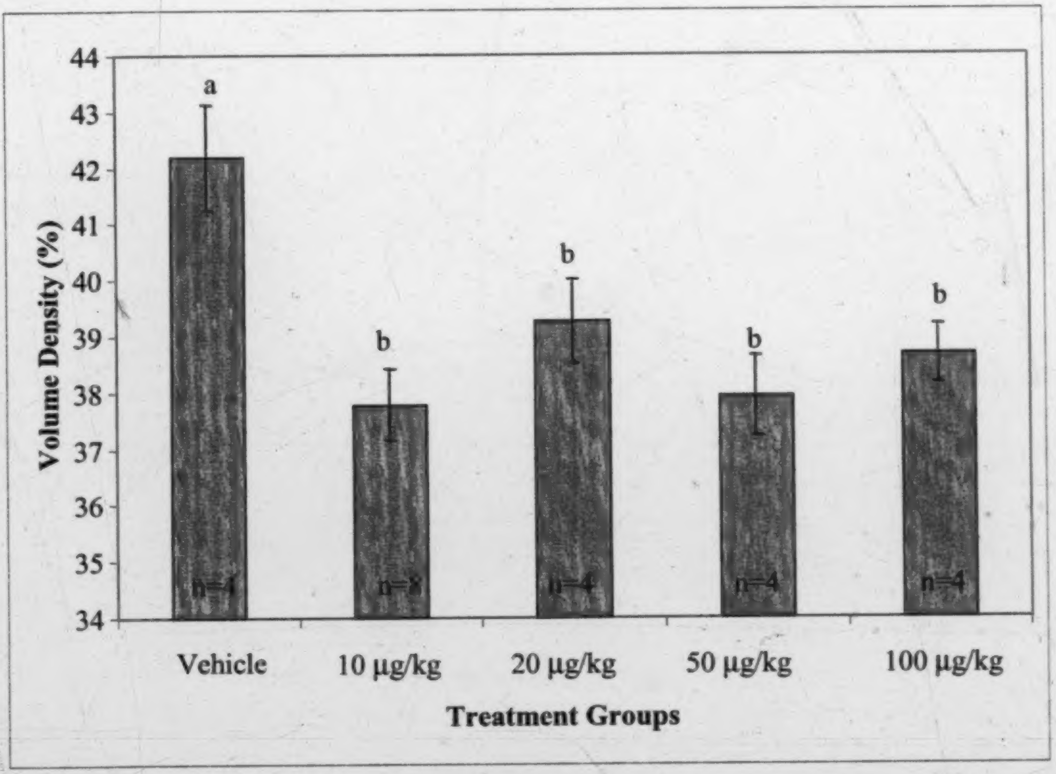

Figure 4.21. Volume density of septa for the LTC$_4$ concentration response treatment groups.

The measured volume density of the septa of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in septa in all four treatment groups as compared to control.

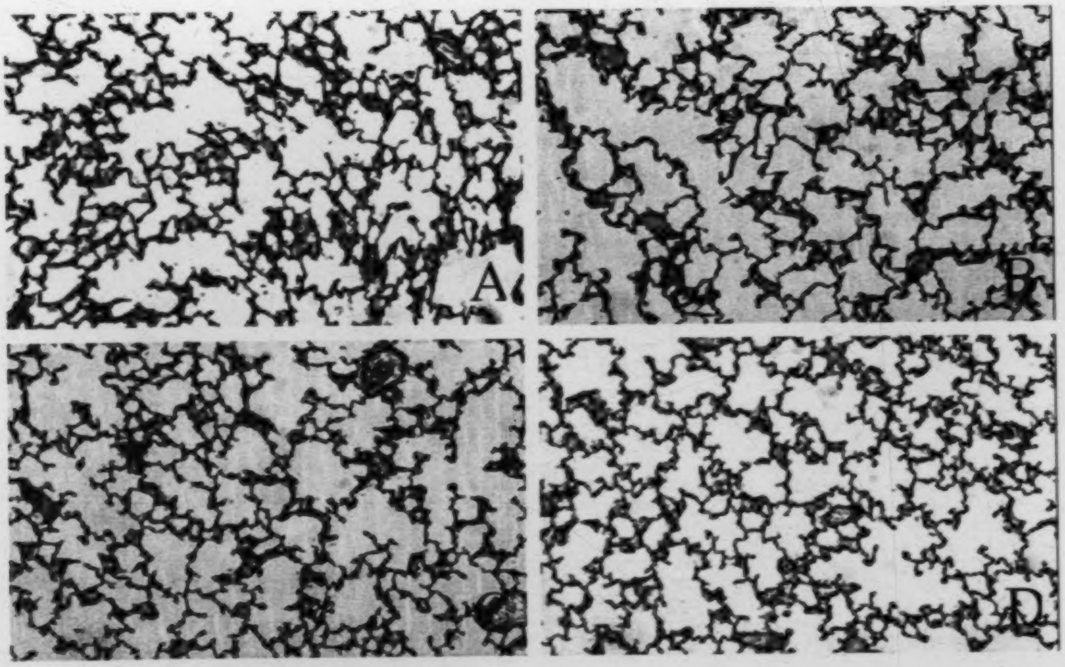

Figure 4.22. Micrographs of the LTC_4 concentration response treatment groups using the 20x objective.

Treatment groups at the 20x objective. *A:* vehicle, *B:* 20 µg/kg; *C:* 50 µg/kg; and D: 100 µg/kg. There were larger airspaces in all three treatment groups as compared to control.

4.2 STUDY 2 Administration of MK-571 to Newborn Rat Pups in both Normoxia and Hyperoxia

MK-571 is a CysLT$_1$ antagonist that blocks the effects of LTD$_4$. It was administered to newborn rat pups in hyperoxia to prevent the inhibition of alveolarization caused by >95% O$_2$. The pups were randomized after birth and placed into two litters of 13 pups. They were placed in chambers from d4-14PN, and injected from d4-13 PN. The treatment groups were air + vehicle (n=7), air + MK-571 (n=6), O$_2$ + vehicle (n=7), and O$_2$ + MK-571.

The initial and final body weights were examined (Table 4.5), and there were no differences. There were no changes in body weight between the treatment groups as compared to control (Figure 4.23).

Morphometric analysis was performed on seven sections from each tissue. There was a significant increase ($p<0.05$) in the volume density of airspace of the three treatment groups when compared to control, and the power was significant (0.988) (Figure 4.24). For the volume density of septa there was a significant decrease ($p<0.05$) in the air + MK-571 and the O$_2$ + vehicle groups as compared to vehicle control, with sufficient power (0.993) (Figure 4.25). No differences were discovered in the volume densities of the bronchi and pleura (data not shown); however, there were differences in vessels (Figure 4.26). There was a significant increase ($p<0.05$) in the air + MK-571 group as compared to the O$_2$ + vehicle group. The photographs taken using the 20x objective clearly demonstrate the morphometric data. (Figure 4.27) All three treatment groups demonstrate increased airspaces as compared to vehicle control. Also, there were few septal buds in the hyperoxic groups. An interesting observation is the similarity in alveolarization between the micrographs for the air + MK-571 and O$_2$ + MK-571 groups.

Treatment	**Initial Body Weight (g)**	**Final Body Weight (g)**
Air + Vehicle	9.3 ± 0.2	26.1 ± 0.7
Air + MK-571	9.2 ± 0.3	25.3 ± 0.6
O_2 + Vehicle	9.1 ± 0.3	25.8 ± 0.7
O_2 + MK-571	9.1 ± 0.3	24.8 ± 0.8

Table 4.5. Initial and final body weights from the MK-571 study.

The newborn rat pups were administered either vehicle or 25 µg/kg of MK-571, and weighed from d4-14 PN. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no statistical differences among the groups for either the initial or final day.

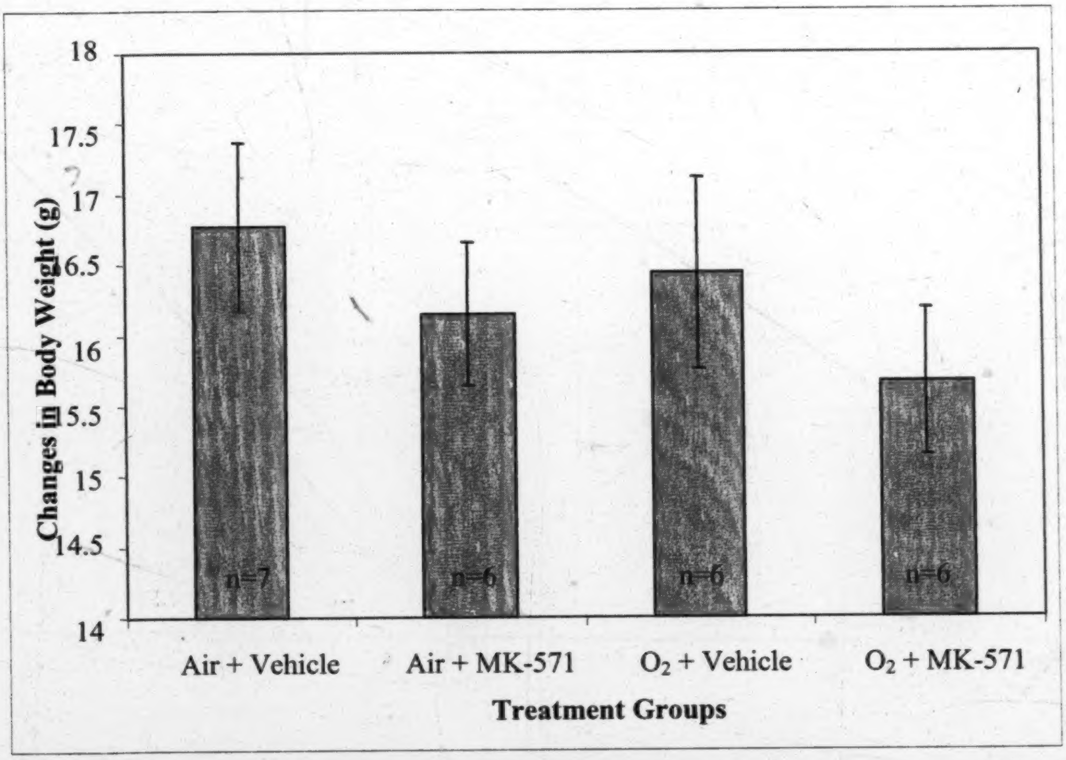

Figure 4.23. Changes in body weight for the MK-571 treatment groups.

The newborn rat pups were weighed from d4-14 PN. The changes in body weight were determined by subtracting the initial from the final body weight. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no changes in body weight due to treatment.

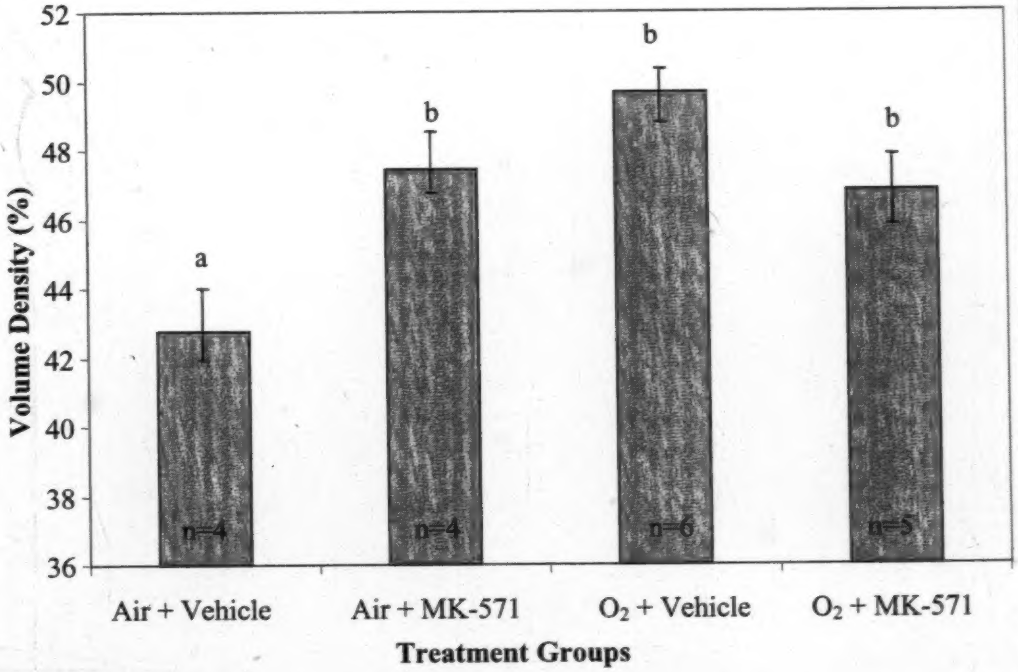

Figure 4.24. Volume density of airspace for the MK-571 treatment groups.

The measured volume density of the airspaces of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase in all three treatment groups as compared to air + vehicle control.

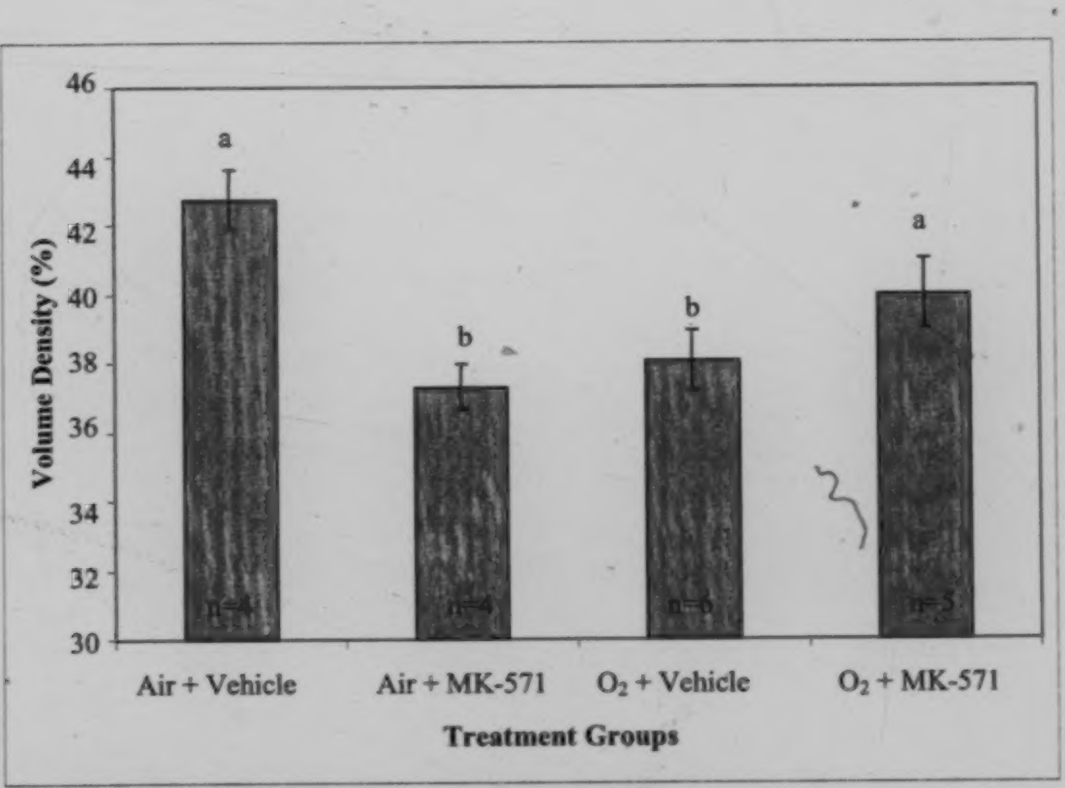

Figure 4.25. Volume density of septa for the MK-571 treatment groups.

The measured volume density of the septa of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in the air + MK-571 and O_2 + vehicle and the other two groups.

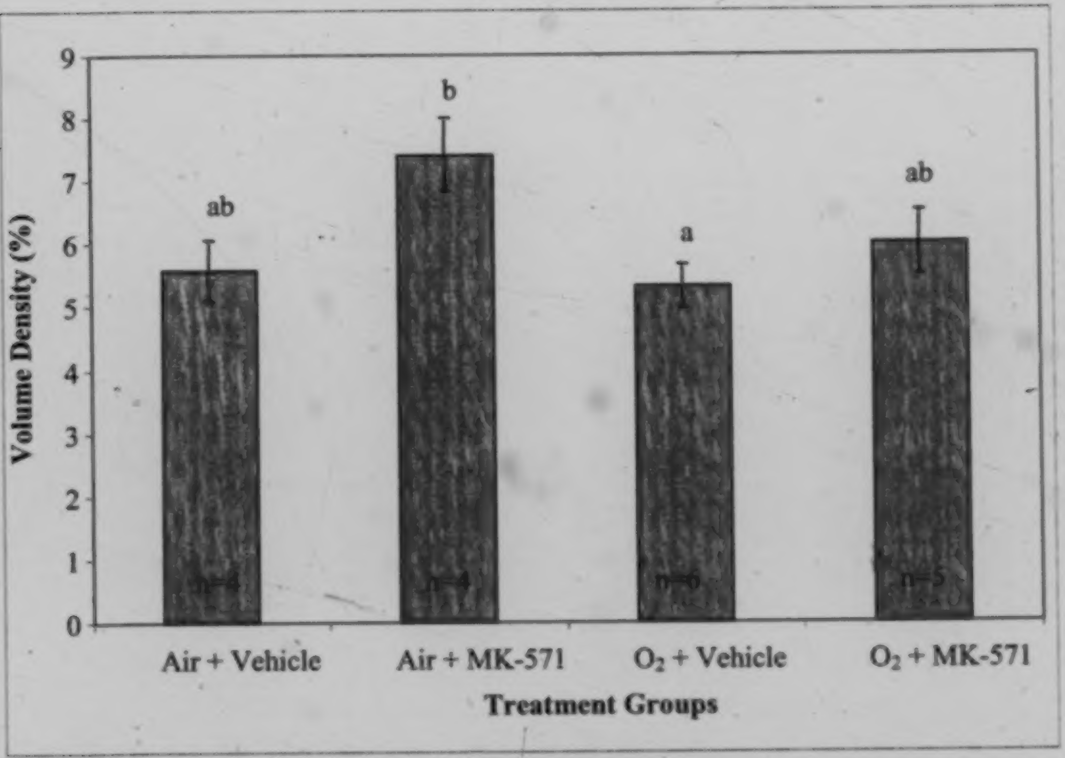

Figure 4.26. Volume density of vessels for the MK-571 treatment groups.

The measured volume density of the vessels of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase in the amount of vessels in the air + MK-571 as compared to the O_2 + vehicle group.

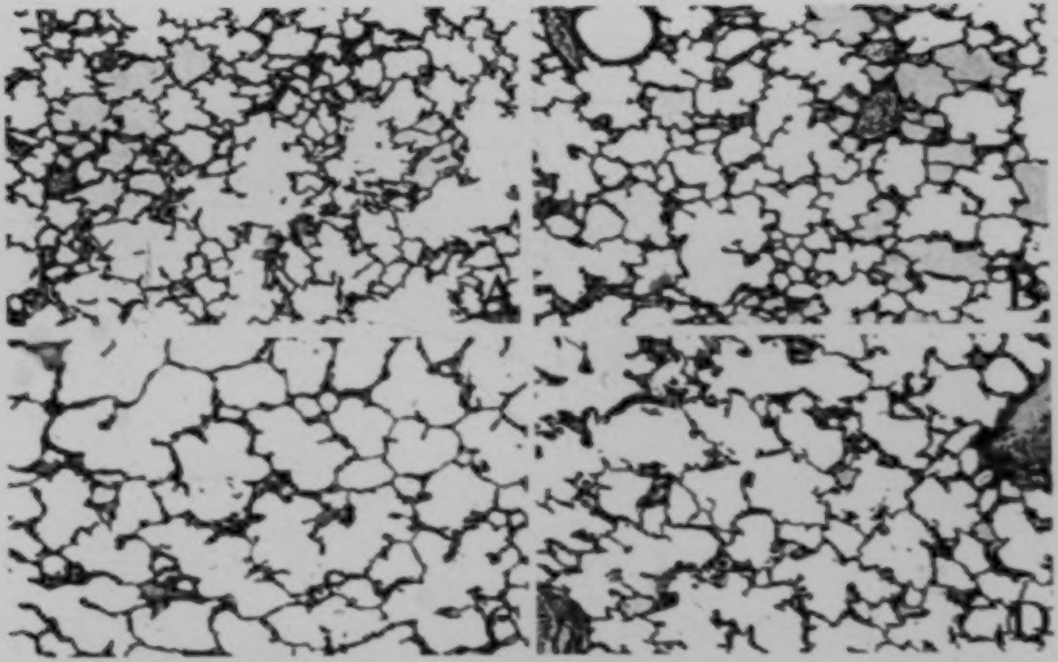

Figure 4.27. Micrographs of the MK-571 treatment groups using the 20x objective.

Treatment groups at the 20x objective. *A:* air + vehicle, *B:* air + MK-571; *C:* O_2 + vehicle; and D: O_2 + MK-571. Using this objective it was apparent that there were larger airspaces in all three treatment groups as compared to control.

5.1 STUDY 1 Administration of Exogenous Leukotrienes to Newborn Rat Pups in Normoxia

Hyperoxia causes lung damage through inflammation and the release of mediators that lead to lung damage. These mediators can be released from leukocytes and macrophages to propagate the effects of inflammation leading to bronchoconstriction and edema, two characteristics of both asthma and BPD (34). Previous studies from our laboratory have demonstrated that the LTs are mediators of the inhibition of alveolarization due to hyperoxia (16). The ROS formed in hyperoxia act on the polyunsaturated fatty acid side chains of membrane limits to cause the peroxidation of the lipids (145). To replicate the hyperoxic effect, exogenous LTs were administered to newborn rat pups in normoxia. We observed the changes in body weight, lung volume, organ weight, and morphometry due to treatment.

5.1.1 Comparison of Lung Development Determinations

Three methods were used to determine the effect of treatments upon changes in lung development. These methods span from classical means of studying lung development changes to modern morphometric analysis. They are summarized in the table below.

The determination of lung volume is a non-specific test of the overall change in total lung air plus tissue volume. This method is not used to determine specific changes or differentiations between lung parenchyma and airspace volume. It is observed from Table 5.1 that there is considerable homology between the values of lung volume in each case (with the exception of the MK-571 treatment as explained in the table legend). Indeed, when lung volume is normalized to body mass (LV/BW ratio) the homology between vehicle treatments is lost and much variation exists. This observation suggests that lung volume (and presumably lung growth and development) is somewhat independent of body growth. Without further testing it is not possible with these data to suggest how independent lung volume is of body mass.

Another means of determining lung development is with modern morphometric analysis. This technique uses the lung as its own normalization standard as specific treatment effects upon lung airspace volume density, septal volume density, and other non-parenchymal volume densities are compared against their aggregate total. This method eliminates other normalizing procedures that would not be directly related to lung development as was observed for body mass, and focuses attention upon treatment effects on specific lung parameters. The sum of the volume densities of the parameters is used as the normalizing parameter, which differs from other morphometric techniques that determine absolute quantities of lung sub-parameters in the degree of change due to treatment effect. Therefore, changes in specific treatment effects are more subtle when expressed as percentages of the aggregate total. For example hyperoxic treatment from d4-14 caused only a 6.9% increase (42.7 ± 1.2 units in vehicle control lungs vs. 49.6 ± 0.7 units in hyperoxia-treated lungs) in the volume density of airspace (Fig. 4.24) as compared to the changes in airspace unit volume (a similar determination) observed by Burghardt (21) which were 0.54 ± 0.09 units in normoxia and 0.92 ± 0.25 units in hyperoxic lungs or a large 70% increase. In this discussion, the volume density measurements will provide the greatest detail about specific changes in the lung as a result of the treatments.

5.1.2 Effect of Exogenous Leukotrienes- Open Chest

The purpose of the study was to administrate exogenous LTs to newborn rat pups in normoxia to determine if this could replicate the effects of hyperoxia on alveolarization.

There was an increase in lung volume in the LTC_4 treated animals compared to control (Figure 4.2). To determine the lung volumes, the lobes were lowered into 4% paraformaldehyde on a balance. The weight changes were directly proportional to the amount of perfusate in the lung. The greater the volume, the greater the amount of perfusate in the lung, which indicated increased airspaces in the lung. LTC_4 lungs had an increased lung volume, therefore they have larger airspaces as compared to control lungs. The volume of these lungs was greater, and there was an associated increase in the percent body weight of the LTC_4 lungs (Figure 4.3). The power of the ANOVA was too low, but this can be explained by the small sample size. The purpose of this study was to perform morphometric analysis on the lungs from each treatment group. The body weights and lung volumes were measured as secondary parameters. Therefore, a power was not determined in advance for the body weights, and the sample size was not large enough.

5.1.3 Effect of Exogenous Leukotrienes on Organ Weights

The subcutaneous route was used to administer the exogenous LTs, therefore the LTs could act on other organs, and thereby indirectly alter lung development via a secondary mechanism. A simple means to assess such a possibility is to weigh other organs, as the most likely effect of LTs would be to cause edema and inflammation (126). Organs were removed and weighed to determine if the LTs caused edema in other tissues that may have then led to lung damage. The organs that were removed were the lungs, liver, heart, right kidney, spleen and thymus; and were selected because they contain CysLT receptors (46). LT administration did alter body weights because LTs in the previous study also altered the body weights.

There was a significant change ($p<0.05$) in body weight between the LTC_4 and LTD_4 groups (Figure 4.4). This was similar to the data of the closed chest exogenous LT group, but not the open chest group. This might be due to the feeding patterns of the individual pups and mothers used in those studies.

There were no differences in the weights of the lungs, hearts, right kidneys, spleens and thymus of the pups due to LT treatment (Figure 4.5). Only the liver weights showed differences, which were increased in the pups receiving LTD_4. This was also demonstrated as a percent body weight. These data demonstrate that the effects of the LTs were direct on the lung, and did not act by secondary mechanisms.

LTD_4 is cleaved by membrane bound dipeptidase, which deactivates it by removing glycine to form LTE_4 (52). A previous study demonstrated that this dipeptidase was produced in small amounts in the newborn liver (52); therefore, LTD_4 may not be metabolized. This would cause an increased amount of LTD_4 that could stimulate an inflammatory response in the liver and induce hepatic edema, causing the mass to increase.

5.1.4 Treated Tissues with Exogenous Leukotrienes- Closed Chest

Changes were made to the laboratory protocol, and the study was repeated with a closed chest. The previous method of lung perfusions and morphometry provided data that did not reflect the *in situ* physiological condition. Our laboratory began using a protocol established by Zeltner *et al.* (151). The lungs were perfused with a closed chest, which ensured that the lungs did not hyperinflate and have enlarged airspaces that are not due to treatment. Morphometric data from open chest tissues would not accurately represent the lung in its normal physiological state.

There were significant differences ($p<0.05$) in the final body weights between all three controls, and from highest to lowest these were: vehicle, No Tx, and no handling (Figure 4.7). Increased handling appeared to decrease the body weights, which indicated that the animals responded to the stress of handling.

The study began with randomization techniques that are a part of the standard protocol from our laboratory. The pups were randomized within an hour after birth into groups of 10. The dams were rotated daily between groups to minimize the effects of lactation from different dams. Also, the rats were almost genetically identical, therefore there should be no differences between the pups. However, the initial body weights of one study (d4 PN) were significantly different ($p<0.05$) (Table 4.3), and since protocol was followed this must be due to chance. The final body weights were also significantly different ($p<0.05$) (Table 4.3), and this may reflect the initial differences.

During the lung perfusions, in all of the studies, there were damaged tissues that were not included. This explains the smaller n number than the body weight data. The lung volumes were determined for each treatment group, and there was a significant decrease ($p<0.05$) in the LTD_4 group as compared to the LTB_4 group (Figure 4.9). There was a significant decrease ($p<0.05$) in the % body weight of the lung in the LTD_4 group as compared to the vehicle control group (Figure 4.10). The % body weight of the lungs was significantly different ($p<0.05$) because the final body weight of the LTD_4 group was not decreased but the lung volume was decreased. There was a significant increase ($p<0.05$) in the lung volume but no change in the % body weight, because the final body weight was also increased in the LTB_4 group.

In the previous morphometric technique used by our laboratory, 10 random areas per tissue section were analyzed per animal. The mean airspace unit volume, the ratio of airspace perimeter to area, volume density of parenchymal tissue, mean septal thickness, and specific internal surface area were all measured (98). However, morphometric techniques established by experts in the field have demonstrated that these measurements were not representative of the whole lung. Zeltner *et al.* determined that one lobe was the smallest sampling unit required to represent the rat lung using light microscopy analysis (151). The volume fraction of parenchyma was equally distributed between all of the lung lobes (151); therefore, any lobe can be used to accurately represent the lung. Another shortcoming of the previous technique was that all analyses were performed by a computer program, and not by a trained observer. A computer does not possess the ability to differentiate between structures; for example, it cannot differentiate between the non-parenchymal airspace in bronchi and parenchymal airspace. It performs counts based on pixel densities, and is unable to differentiate between debris in the lungs and tissue like bronchi walls, pleura, and septa. For this reason, it was more appropriate that a human observer perform the analysis.

Previous studies in our laboratory produced results that are much too large to accurately represent the small changes associated with inhibited alveolarization. This was due to the bias of the study as the observer selected areas that they felt most accurately represented their data requirements. They also used sections of the lung from the hilus area where there are larger airspaces as compared to the periphery. Their results suggested a two-fold increase in the mean airspace volume unit (21), which is much too high. The changes associated with alveolarization are much more subtle than their results would indicate. The new morphometric technique we demonstrated a 7% increase in the volume density of airspace between the hyperoxic and normoxic animals (Figure 4.24). This difference was still significant, but more realistic than the previous results from our laboratory in representing the changes in alveolarization due to hyperoxia.

The new technique was developed in the rat by Zeltner and Burri (152). Analysis was performed on seven sections spaced equally through the right lung lobe. Point count analysis was used, which involves a target traveling across the tissue, using a motorized stage, following a grid established by ImagePro Plus software. There must be 1000 targets hit per tissue to make an accurate assessment.

Each lung lobe contains 80-85% parenchymal, and 15-20% non-parenchymal tissue, and all data must demonstrate this to be representative of the entire lung (24). The parenchyma is composed of the airspaces and septa, including capillaries (27). The non-parenchymal tissues are the bronchi and bronchioles, blood vessels (excluding interalveolar capillaries), and pleura (27). The measurements from the vehicle control group confirmed that, by using the right caudal lobe, this technique accurately represented the entire lung (Figure 4.11). The volume densities of the parenchymal tissues of both the vehicle control and the No Tx groups were compared (Figure 4.12) and there were no differences, therefore for the rest of this study the No Tx group was not included.

There was a significant decrease ($p<0.05$) in the volume density of airspace in the LTD_4 group as compared to the other groups (Figure 4.13). The LTB_4 and LTC_4 treatment groups appeared to cause an increase in airspace; however, this was not significant. It was expected that the volume density of septa data should complement the volume density of airspaces. If there was an increase in airspace, than there should be a corresponding decrease in septa, which occurred in our data (Figure 4.14). There was a significant increase ($p<0.05$) in the volume density of septa in the LTD_4 group as compared to the other three groups. Also, there was a significant decrease ($p<0.05$) in the LTB_4 group when compared to control. The LTC_4 group appeared to be decreased as compared to vehicle control; however, this was not significant. There were no changes in the volume densities of the bronchi and pleura; however, there were significant differences ($p<0.05$) in the vessels (Figure 4.15). There was a significant increase ($p<0.05$) in the LTB_4 group as compared to control. The increase in the LTB_4 accounts for the differences in septa but not in airspace.

The hypothesis was that the exogenous LTs would replicate the effects of hyperoxia by inhibiting alveolarization. Previous studies from our laboratory demonstrated that hyperoxia increased airspace and decreased septa (21). This was demonstrated by exogenous LTB_4 and LTC_4, although it did not always reach significance. This may be explained by a few different scenarios. First, hyperoxia may increase not only the LTs but also their receptors. This would explain why the inhibition was not as severe as was previously observed in hyperoxia (16). Second, the concentration (10 μg/kg) may not have caused the maximal effects, and for this reason a concentration response was later performed. And finally, many mediators (not only the LTs) were released during hyperoxic exposure that also inhibited septation. In the absence of the other mediators the inhibition would not be as extensive.

In the LTB_4 and LTC_4 treatment groups, septal thinning was also apparent, suggesting that these LTs might accelerate lung maturation. This may involve remodelling of the extracellular matrix. LTC_4 has been demonstrated to affect the matrix metalloproteinases (MMPs) (59).

After hyperoxic exposure rat lungs have increased edema, which results in increased blood cells in the alveoli (32). In the lungs of infants with BPD there is an increased number of neutrophils, and edema was also demonstrated in the LTB_4 and LTC_4 groups of the present studies (51). This has previously been demonstrated in the presence of LTC_4, where there was increased migration of eosinophils into the airways (91). The gap junctions between endothelial cells widen in the presence of CysLTs, which causes edema (75).

Newborn rats in hyperoxia have increased numbers of neutrophils in their lungs (40, 145). LTB_4 stimulates the transmigration of neutrophils from the capillaries into the airspaces to propagate the inflammatory response (36). Neutrophils release AA and generate O_2^- (138), which cause more damage to the lung. The CysLTs also release AA from cells, which releases more inflammatory mediators (5), including cytokines, chemokines, and MMPs (115).

The results from the administration of 10 μg/kg of LTD_4 were unexpected. Previous studies from our laboratory used an ELISA to determine that an inhibition in alveolarization is related to an increased amount of CysLTs (16); however, they did not examined the effects of the individual CysLTs. It was assumed that LTC_4 and LTD_4 would cause similar effects to lung tissue; however, this did not occur. The decreased volume density of airspace and the increased volume density of septa suggested that LTD_4 was driving septation, and therefore, accelerating alveolarization. This introduced a novel and intriguing possibility: is LTD_4 be involved in normal lung development?

There are a variety of mechanisms that might be at work in the inhibition of alveolarization. Perhaps γ-glutamyl transpeptidase was deactivated, and therefore could not cleave the peptide side chain and convert LTC_4 to LTD_4. A second possibility was that the formation of LTC_4 and its transport by MRP1 out of the cell was increased, and there was always a greater amount of LTC_4, and not LTD_4, in the extracellular environment. Another explanation could be that LTD_4 was quickly being converted to LTE_4 and deactivated by increased amounts of membrane-bound dipeptidase. And finally, hyperoxia could be up-regulating the CysLT receptors.

The LTD_4 tissues appeared to have thickened septa. Lung injury has been associated with fibrin deposition in the septa (145), and may have occurred in the LTD_4 treated lungs. The CysLTs cause fibroblasts to proliferate and increase collagen synthesis (59). Hyperoxia has also been shown to affect the elastin fiber structure and composition (16).

In asthma, LTs cause a thickening of the ASM, which results in airway hyperresponsiveness (20). The ASMs do not produce CysLTs, and the location of CysLT synthesis requires further investigation (55). The endothelium releases mediators that can act on ASM cells (85), and perhaps the CysLTs were these mediators, which then caused thickening and hyperreactivity.

Previous studies have demonstrated that eicosanoids promote angiogenesis (45). Both LTC_4 and LTD_4 were angiogenic in the chorio-allantoic membrane in the developing chick (141); and they may also be angiogenic in the human lung. Their respective effects on the developing endothelium may help interpret the results of this study. LTC_4 may stimulate the fusing of the double capillaries into the single capillary before the septal bud has elongated, thereby inhibiting septation. It was most likely the $CysLT_2$ receptor causing this effect, since LTC_4 decreased the volume density of the septa. Alternatively, LTD_4 may cause increased proliferation and migration of the endothelium. There appeared to be an increased amount of blood vessels in the LTD_4 treated lungs, and this requires further investigation.

Alveolarization is composed of two separate processes, alveogenesis and angiogenesis, which must work together for normal lung development to occur. If one process is inhibited, this will cause an inhibition of the other will occur. In our studies, LTD_4 appeared to drive alveogenesis, while LTB_4 and LTC_4 may have inhibited angiogenesis. The inhibition of angiogenesis would cause a decrease in the amount of capillaries available to support the developing septal tissue. Although LTD_4 is increasing the amount of septal budding and elongation; if there are no vessels to support them, they will atrophy and thin. We suspect that this mechanism explains the enlarged airspaces demonstrated in hyperoxic lungs.

Intestinal epithelial cells produced LTs, mainly LTB_4 and LTD_4, to ensure their survival during inflammation (112). This indicated that the LTs may act in a paracrine and autocrine fashion, and that the cells could express the receptors. Perhaps the alveolar epithelial cells also possess this survival mechanism. Currently, these cells are not thought to produce LTs or possess their receptors.

The LTs have roles in other organs, such as the skin where they are involved in the pathogenesis of systemic sclerosis skin lesions (86). When administered exogenously they have been shown to increase the arterial pressure in the pulmonary vasculature in various animal models, including rats (7). It has been shown that LTC_4 and LTD_4, not LTE_4, cause the contraction of these pulmonary arteries (7).

BPD lungs have enlarged airspaces and thick-walled alveoli (99). The LTC_4 lungs from our study contained the enlarged airspace, while the thick-walled alveoli were observed in the LTD_4 treated lungs. The pulmonary vasculature was inhibited in BPD, potentially by decreased amounts of either angiogenic factors or the receptors of these factors (11).

In the clinical setting BPD lungs were characterized by bronchiole and septal fibrosis; however, many models of the disease have not demonstrated these features (119). There is evidence that the CysLTs are involved in pulmonary fibrosis, which is a characteristic of BPD (59). From our results it was the tissues treated with LTD_4 that caused a thickening of the septa, perhaps it was this CysLT that propagates pulmonary fibrosis in BPD.

Hyperoxia produces ROS, and stimulate the production of AA metabolites (134). The 5-LO pathway also produces ROS, which increases the number of ROS in the lung.

This effect was hard to replicate with the administration of the exogenous LTs. While the lungs would be damaged by the ROS from the AA metabolites, they would not have those that were directly formed from the hyperoxia. Therefore, the administration of exogenous LTs would not completely replicate the effects of hyperoxia on the inhibition of alveolarization.

5.1.5 Concentration Response of LTC_4

The purpose of this study was to determine the concentration of LTC_4 that caused the greatest inhibition of alveolarization.

The lung volumes (Figure 4.18) measured from this study were similar to those from the other studies performed. The control data of this study in the % body weights varied between groups more than the experimental data (Figure 4.19), and was lower than what was previously measured, but this can be explained by the final body weights. The pups from this study were born earlier than normal, as demonstrated by their increased initial and final body weights (Table 4.4). We suspect that at the end of the alveolar stage the lung volume reaches a plateau, whereas the body weights are still steadily increasing. This may explain the difference in the % body weight of this study as compared to others. A previous student from my laboratory discussed the results of her studies with a biostatistician, who determined that each experimental group should be compared to its control and not to groups from other experiments. Therefore, the % body weight results of this study, although different from the other studies, were included in this thesis.

Morphometric analysis was then performed on seven sections per tissue. The data from the 10 μg/kg group were included to determine the differences between all of the concentrations. There was a steady increase in the volume density of airspace in the treatment groups as compared to control, and this reached significance ($p<0.05$) with the 50 µg/kg group (Figure 4.20). There was a significant decrease ($p<0.05$) in the volume density of septa of all treatment groups as compared to control (Figure 4.21). These data demonstrated that the most potent concentration of LTC_4 in the inhibition of alveolarization was 50 µg/kg.

5.2 STUDY 2. Administration of MK-571 to Newborn Rat Pups in both Normoxia and Hyperoxia

The original hypothesis for this study was that the administration of the $CysLT_1$ antagonist, MK-571, would block the inhibition of alveolarization from hyperoxia mediated by the LTs. However, the data from the exogenous LT study demonstrated that LTD_4, the CysLT with the highest affinity to $CysLT_1$, was not an inhibitor of alveolarization. Instead, it appeared to drive septation, and accelerate alveolarization. Therefore, the objective of the study must be altered to account for this new data. LTD_4 caused an acceleration of alveolarization in normoxia, and by using a blocker this could be prevented. We also predict that in LTD_4 stimulates abnormal septation in hyperoxia, which would be blocked by MK-571.

The dams were rotated between the normoxic and hyperoxic chambers daily to prevent any hyperoxic damage that may effect lactation, as hyperoxia is cytotoxic (107). Previous studies have demonstrated that dams in >95% O_2 were healthy for the first 48 h of exposure; however, they soon developed pulmonary edema and respiratory failure that lead to death after 72 to 96 h of exposure (83). One pup in the hyperoxic study died on d12 PN, which was not unexpected due to the stresses placed on the animal.

There was an increased volume density of airspace associated with air + MK-571, which was consistent with the results of Study 1. LTD_4 stimulated septation, and MK-571 blocked this action. Therefore, when LTD_4 was inhibited with MK-571 in normoxia there would be decreased septation and increased airspaces. This again suggests a role for LTD_4 in normal alveolarization.

Another interesting observation was the decreased inhibition of airspace of the O_2 + MK-571 group as compared to the O_2 + vehicle group. The $CysLT_1$ receptor was acted upon by not only LTD_4 but also LTC_4. Therefore, if the receptor was blocked the inhibition of septation caused by LTC_4 would also be blocked. Also, the activation of the $CysLT_1$ stimulates the production of more CysLTs, which can act on cells to produce cytokines, chemokines, and MMPs to potentiate their effects (115). If the receptor was blocked then there would not be an increase in mediators; and therefore the inhibition of alveolarization would not be as severe. $CysLT_1$ can also be upregulated by cytokines (68), and if it was blocked this action would not occur.

There was a significant increase ($p<0.05$) in the volume density of airspace in all three treatment groups as compared to control (Figure 4.24). There were significant decreases ($p<0.05$) in the volume density of septa in the air + MK-571 and the O_2 + vehicle groups as compared to control (Figure 4.25). The O_2 + MK-571 group had a significant difference ($p<0.05$) in airspace but not in septa, and may be explained by the volume density of the vessels (Figure 4.26). There was a significant increase ($p<0.05$) in the air + MK-571 group, with a trend towards an increase in the other two treatment groups as compared to O_2 + vehicle. This result was consistent with previous studies that have demonstrated endothelial cell damage in hyperoxia (11).

A previous study demonstrated that lungs with increased LTs due to hyperoxia contained increased amounts of ASM (20), which was similar to airway remodelling in asthmatic lungs. This demonstrates another link between the morphology of BPD and asthma, and may provide more evidence that LT antagonists may be used in infants with BPD.

In our analysis there was no distinction between bronchial walls and lumina; therefore, the effects of MK-571 on the bronchi was not determined. There were no differences in the number of bronchi; however, their walls appeared to be thicker.

Currently only $CysLT_1$ antagonists are being used in the treatment of asthma, and since BPD has a similar etiology to this disease our laboratory began studying these antagonists with our newborn rat model of BPD. However, the results were not expected. The administration of exogenous LTs demonstrated that the CysLTs have different effects on the lung, and LTD_4 did not inhibit alveolarization. It was LTC_4 that caused the inhibition, and since it acts primarily on the $CysLT_2$ receptor, antagonists for this receptor should be studied. The only blocker for this receptor is BAYu9773, although it is not selective and acts on both CysLT receptors. The data suggest that MK-571 antagonists should not be used to treat newborn lungs at risk of BPD. This is because it was demonstrated that it inhibits septation, and therefore might have adverse effects.

5.3 IMPLICATIONS OF THIS RESEARCH

The clinical implications of this research involve the human chronic preterm lung disease, BPD. Preterm infants which are born weighing <1000g and before 28 weeks gestation are born before alveolarization has begun (38, 144). Therefore, these lungs are underdeveloped and cannot support the gas-exchange needs of these infants. BPD is a clinical disorder involving permanent lung damage due to barotrauma and oxygen damage, which occurs when preterm infants are placed on a ventilator (78). BPD lungs have increased levels of LTs, which mediate the oxygen damage caused by the ROS (50). Previous studies have demonstrated that LTs mediate many of the clinical features associated with asthma such as increased vascular permeability, eosinophil infiltration and bronchoconstriction (13).

It was also shown that the CysLT$_1$ receptor antagonist MK-571 would block these effects (13), all of which are symptoms of both asthma and BPD. Many LT antagonists (montelukast, zafirlukast) have anti-bronchoconstrictive and anti-inflammatory actions, and are currently on the market as treatment for asthma (57). Therefore, there is a possibility that these anti-LT drugs may be administered to treat BPD. To date the drug is only used on patients over two years of age, although it is well tolerated and does not exhibit harmful effects when compared to control (79). The long-term objective of these studies is to determine if LT antagonists can be administered to preterm infants to prevent the oxygen damage that is mediated by the LTs. Although LT antagonists may not be extremely effective in pediatric asthma, currently there are no treatments for BPD. Even if they could be effective for a small percentage of preterm infants afflicted with this disease this would be an improvement over the current situation.

We administered the CysLT$_1$ antagonist MK-571 to our newborn rat model of hyperoxic lung damage. LTD$_4$ has the highest affinity to this receptor, and therefore it was the effects of this LT that were primarily blocked. We determined that LTD$_4$ is involved in normal lung damage, and when MK-571 was administered to pups in normoxia it caused damage similar to hyperoxic damage. We concluded that without further studies, this antagonist should not be used to treat preterm infants with BPD since it inhibits normal lung development.

Our results suggest that the inhibition of angiogenesis by LTB_4 and LTC_4 causes an arrest in alveogenesis, which inhibits normal lung development. This suggests that antagonists for the $CysLT_2$ receptor and the two BLT receptor may prevent this inhibition. The results of these studies using our hyperoxic lung damage model may lead to clinical trials on preterm infants on ventilators to determine if they could prevent the lung damage mediated by LTs.

5.4 SUMMARY

Previous studies from our laboratory have demonstrated that the LTs are mediators in hyperoxic lung injury (16, 21, 62). First, a model of BPD was established using newborn rats that demonstrated that hyperoxia inhibits alveolarization. Second, lungs from these rats had elevated levels of LTs. And finally, 5-LO and FLAP inhibitors (Wy-50295 and MK-0591) blocked the inhibition of alveolarization. The data illustrated that the LTs may be mediators in the hyperoxic inhibition of alveolarization.

Study 1 was the final step required to prove that the LTs were mediators of this inhibition. If the administration of exogenous LTs in the absence of hyperoxia caused an inhibition of alveolarization, this would prove their role as mediators. The hypothesis was that both LTB_4 and the CysLTs would replicate the effect of hyperoxia and cause the inhibition. Our hypothesis was confirmed with the administration of LTB_4 and LTC_4. They both caused an increase in the volume density of airspace and a decrease in the volume density of septa, which are characteristics of hyperoxic lung injury.

Alternatively, LTD_4 increased the volume density of septa and decreased the airspace. This demonstrated that LTD_4 might drive septation and stimulate alveolarization. This suggests that LTD_4 may be involved in normal lung development, and when it is administered exogenously it accelerated alveolarization. This was unexpected, as it was previously thought that all CysLTs had the same effect on the lungs. Previous studies from our laboratory used an ELISA sensitive to LTC_4, LTD_4 and LTE_4 to illustrate that the CysLTs as a group mediated the effects of hyperoxia by. It was unexpected that two closely related molecules, LTC_4 and LTD_4, would have opposite effects on the lung tissue. The only difference between the two was the γ-glutamyl on the LTC_4 side chain, which is cleaved to form LTD_4. LTC_4 has a glutathione group attached to it that cause the inhibition of angiogenesis, and be the reason for its different effects. We suspect that the difference may be caused by the CysLT receptors, instead of the CysLTs themselves. We speculate that hyperoxia may cause not only an increase in the CysLTs but also in their receptors. LTC_4 and LTD_4 act on the $CysLT_1$ and $CysLT_2$ receptors with different affinities, and perhaps this caused the opposite effects on alveolarization. This was the reason for the ontogeny study of the receptors in study 4.

For the second study the hypothesis was that the administration of 25 μg/kg of MK-571 to newborn rat pups in hyperoxia would prevent the inhibition of alveolarization caused by CysLTs in hyperoxia. MK-571 is an antagonist that blocks the $CysLT_1$ receptor, whose primary agonist is LTD_4. Our studies demonstrated that LTD_4 accelerated alveolarization, and the effects of LTD_4 during hyperoxia are not known. We speculated that it was involved in the fibrosis and thickening of airways. The revised hypothesis was that the administration of MK-571 in hyperoxia might reveal the effects of LTD_4 in hyperoxia.

The data demonstrated that MK-571 decreased the effects of hyperoxia. It decreased the volume density of airspace and increased the volume density of septa. This was perhaps due to the inhibition of the actions of LTC_4, and not LTD_4, on the $CysLT_1$ receptor. LTC_4 caused the inhibition of alveolarization, which would be blocked.

Study 1 suggested that LTD_4 may be involved in normal lung development, and the results from this study gave further evidence to support this data. MK-571, when administered to pups in normoxia, caused significant changes ($p<0.05$) as compared to control in the volume density of airspace. The volume density of septa decreased, although not to significance. This demonstrated that by blocking the actions of LTD_4 with the $CysLT_1$ antagonist MK-571 there was an inhibition in septation. These data confirmed that LTD_4 was involved in normal lung development.

An ontogeny study of the CysLT receptors was started and is ongoing. The hypothesis was that hyperoxia would enhance the amount of the CysLT receptors in newborn rat lungs. The preliminary results indicate that hyperoxia caused a down-regulation of the receptors when compared to control; however, the study is still in the initial stages.

5.7 CONCLUSION

These data demonstrate, for the first time, that LTs both arrest and accelerate alveolarization, and are therefore key mediators in this final stage of lung development. Further, these data suggest that elevated LTB_4 and LTC_4 are associated with hyperoxic lung disease. The observed differences between exogenous LTs and hyperoxic effects may be due to insufficient levels of LT receptors (BLT_1, BLT_2, $CysLT_1$ and $CysLT_2$), and the association of the two components of alveolarization, angiogenesis and alveogenesis. We predict that in the presence of hyperoxia LTB_4 and LTC_4 are inhibiting angiogenesis, whereas LTD_4 may be promoting alveogenesis (Figure 5.1). The inhibition of angiogenesis would cause an overall inhibition of alveolarization. The administration of the $CysLT_1$ receptor antagonist MK-571 added evidence that LTD_4 is involved in normal lung alveolarization. Already there are therapies using LT antagonists in treating asthma, and perhaps in the near future lung diseases like BPD will be treated similarly.

Study	Change in Body Weight (g)	Lung Volume (g)	LV/BW Ratio (%)	Volume Density of Air (%)
Open Chest	17.83 ± 0.35	1.23 ± 0.05	4.47 ± 0.14	ND
Organ Weight	17.94 ± 0.34	ND	ND	ND
Closed Chest	16.33 ± 0.38	1.23 ± 0.03	4.89 ± 0.13	44.3 ± 1.0
LTC_4 Concentration-response	22.10 ± 0.47	1.26 ± 0.05	3.65 ± 0.12	44.8 ± 0.9
MK-571	16.77 ± 0.60	ND	ND	42.7 ± 1.2
LTD_4 Concentration-response	19.15 ± 2.65	1.24 ± 0.07	4.28 ± 0.14	46.5 ± 0.8

Table 5.1. Comparison of body weight, total lung volume, the lung volume/body weight ratio, and the volume density of air in vehicle-treated lungs from each study.

Both the lung volume, as determined by the volume weight method, and the volume density of airspace, as determined by morphometric analysis, are quite consistent between studies. Significant variation occurs in the body weight changes with each study. Since the rather variable body weight is the denominator of the third descriptor, lung volume/body weight ratio in column three, these ratios also vary considerably from study to study. *ND= not determined

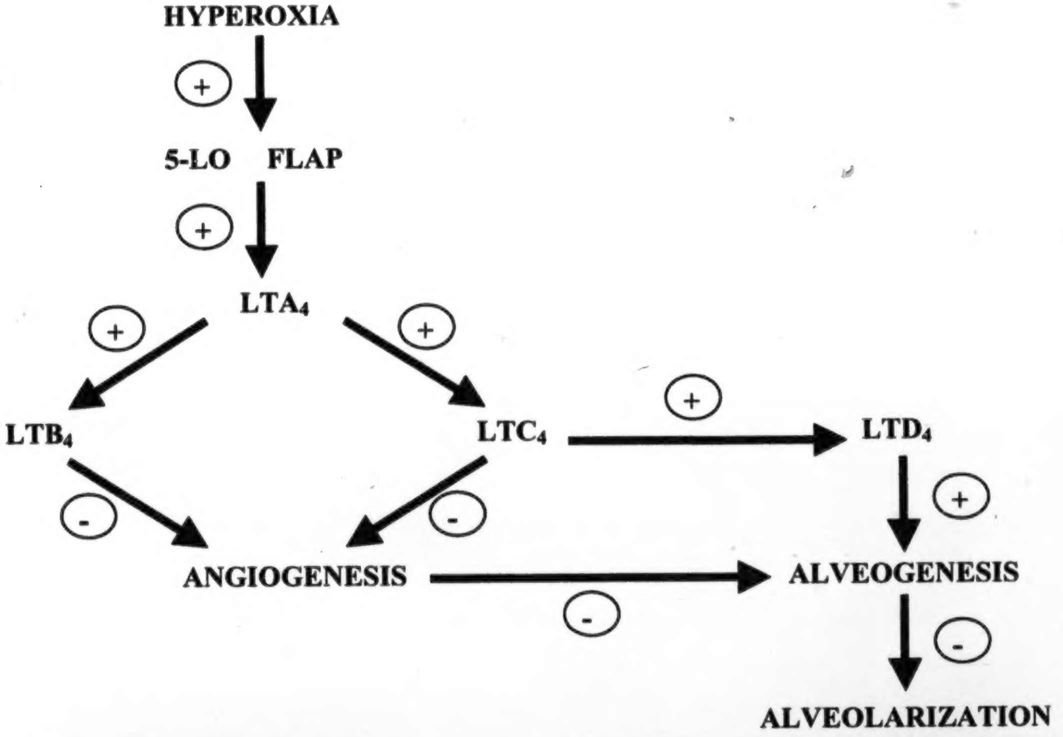

Figure 5.1. Proposed schematic of the role of LTs in the hyperoxic inhibition of alveolarization.

This is the proposed pathway that demonstrates the role of LTs in the hyperoxic inhibition of alveolarization. Previous studies from our lab demonstrated increased levels of 5-LO, FLAP, LTB_4 (62), and CysLTs (21) in hyperoxia. These increases lead us to predict that there would also be an increase in LTA_4 in hyperoxia. LTB_4 and LTC_4 inhibited alveolarization in our studies, and we predict that this was due to an inhibition in angiogenesis. Our first study demonstrated that LTD_4 stimulated septation, which leads us to predict that it will stimulate alveogenesis in hyperoxia. It has been shown that by inhibiting one process of alveolarization both will be inhibited (11), which leads to an arrest in alveolarization. We predict that an inhibition in angiogenesis will inhibit alveogenesis, and ultimately alveolarization.

CHAPTER 6. FUTURE STUDIES

6.1 STUDY 1. ADMINISTRATION OF EXOGENOUS LEUKOTRIENES TO NEWBORN RAT PUPS IN NORMOXIA

The studies performed both answered the hypothesis of the study, and provided new insight into the role of LTs on newborn lung alveolarization. However, it has also created questions that require further study.

The inhibition of alveolarization may be due to arrested of angiogenesis. This may be due to the effects of LTB_4 or LTC_4, since these were the two LTs that inhibited alveolarization in Study 1. Electron microscopy must be performed to examine endothelial cells using morphometric analysis to determine if there are changes in the structure and number of vessels. The treated tissues must also be examined to determine if angiogenic factors are affected.

Previous hyperoxic studies discovered increased septal fibrin deposition in the presence of hyperoxia, which could explain why the LTD_4 tissues had thickened septa. The tissues treated with LTD_4 must be stained to determine if the amount of fibrin is altered due to treatment (145). This will be important in determining the effects of LTD_4 on the parenchymal tissue. Hyperoxia also alters the amount of elastin fibers; therefore, it should be determined if the LTs are mediators of this alteration.

We determined that the LTs had different effects on alveolarization. LTD_4 appeared to drive alveogenesis, while LTB_4 and LTC_4 may have inhibited angiogenesis. The inhibition of angiogenesis would cause a decrease in the amount of capillaries available to support the developing septal tissue. Although LTD_4 is increasing the amount septal budding and elongation, if there are no vessels to support them they will atrophy and thin. Future studies must be carried out on these tissues using TUNEL to determine if there is increased apoptosis in these cells as compared to control. If there was this would provide evidence to our theory of increased alveogenesis being affected by decreased angiogenesis to cause an overall inhibition of alveolarization.

The tissues treated with LTB_4 and LTC_4 had enlarged airspaces and what appeared to be thin septa. Morphometric analysis must be performed on these sections to determine if they are thinner septa due to treatment. If this demonstrates that the septa are thinner, the mechanism behind this must be determined. Zymography might be performed on the lung tissues treated with exogenous LTs to determine if there are increased numbers of MMPs due to treatment.

BPD is characterized by increased edema in the lung tissue, which is replicated in hyperoxia (2, 39). If this effect is replicated by administering exogenous LTs our hypothesis will be supported. The tissues that were treated with exogenous LTs appeared to have increased amounts of blood cells in the alveoli, which is a sign of interalveolar edema. Studies must be performed on these tissues to determine if edema occurred. Also, if more rat pups are treated their lungs should be lavaged to determine the extent of edema and proteinosis caused by the administration of exogenous LTs, and to discover which leukocytes are in abundance in the alveoli in these conditions.

Neutrophil cell count is elevated in both hyperoxic models of lung injury and in infants who develop BPD (40, 51), and is associated with increased levels of LTB_4. To determine if there were increased levels of neutrophils in the treated lungs, the sections should be stained and analyzed using immunohistochemistry.

The enzymatic activity of GGT is increased in hyperoxia; however, the activity of GGL has not been determined (84). It is difficult to differentiate between the two enzymes, therefore knockout mice for GGT must be used to determine the enzymatic activity of GGL in both hyperoxia and normoxia. This will allow us to discover if the protein levels of this enzyme are also increased in hyperoxia.

Also, LTD_4 may have been quickly converted to LTE_4 and deactivated by increased amounts of membrane-bound dipeptidase in hyperoxia. The enzymatic activity of this enzyme must also be determined in both hyperoxia and normoxia.

Instead of affecting the LT synthesis pathway, hyperoxia may be up-regulating the CysLT receptors. This is the reason for the ontogeny study.

6.1.1 Effect of Exogenous Leukotrienes on Organ Weights

The results of this study created questions that must be addressed. There were differences when the lungs were weighed while still wet. Another study may be performed to determine if edema occurred in the other organs besides the lung due to the treatment. This is achieved by drying the organs in an oven to compare the wet and dry weights, and observe the differences. Differences would indicate inflammation and edema.

There were changes in the body weights, but in only one of the organs (liver) observed. There may have been differences in bone density, which therefore should be measured in future pups. Also, ELISAs would confirm if there are increased amounts of CysLTs in those organs.

6.2 STUDY 2. Administration of MK-571 to Newborn Rat Pups in both Normoxia and Hyperoxia

Future studies must be performed using electron microscopy to determine the effects of MK-571 on the bronchi in newborn rat lungs. LTD_4 has been shown to be involved in fibrin deposition and the thickening of septa; therefore, electron microscopy must be performed on the air + MK-571 group to determine if the MK-571 will inhibit septation. This would support that LTD_4 is involved not only in the hyperoxic response, but also in normal lung development. In the past LTs have been suspected to only be involved in the inflammatory response, but perhaps they are also involved in normal lung development.

A future study is required to prove that the dose of MK-571 (25 µg/kg) is effective. Three treatment groups must be used: one vehicle control (H_2O); one group administered 10 µg/kg of LTD_4 (the most effective dose); and a third administered both LTD_4 and MK-571. Morphometric analysis will determine if the MK-571 blocked the effects of LTD_4. A previous study determined that LTD_4 is more potent than MK-571; therefore, a larger amount of MK-571 should be used to effectively block the effects of LTD_4 (122).

The effects of hyperoxia were not blocked by the administration of a LTD_4 inhibitor. This study should be repeated using LTB_4 (BLT_1 and BLT_2) and LTC_4 antagonists to determine if they could prevent the lung damage caused by ROS. To date the LTB_4 antagonists NAC, DPI, PD98059, or CP105696 have been used in mice lungs to prevent neutrophil transmigration, and perhaps they could also be used in our rat model (14). Currently BAY u9773 is the only antagonist available to characterize the $CysLT_2$ receptor (7).

CHAPTER 7. REFERENCES

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APPENDICES

A.1 CONCENTRATION RESPONSE OF LTD_4

A.1.1 Results

Newborn rat pups were injected with either vehicle, or LTD_4 at 20, 50 or 100 μg/kg. Although a small sample size was used, the main intent of this study was to perform morphometric analysis to determine which concentration of LTD_4 was most potent. Therefore the number of replicates was small for each dose of LT.

The body weights, lung volumes, and percent body weight was examined to determine if there were any trends due to treatment. Therefore, the powers of these tests were lower than 0.800 (0.05-0.221). The initial and final body weights of the treatment groups were determined, and there were no differences (Table A.1). The changes in body weight were calculated by subtracting the final by the initial weight (Figure A.1), and no differences were found. The lung volumes and % body weight were also calculated (Figures A.2 and A.3), and there were no changes due to treatment.

Morphometric analysis was performed to determine if there was a concentration response when exogenous LTD_4 was administered. Only one tissue was processed for analysis for the 20 μg/kg group, and these results were not included. For the volume density of airspace, there was a significant decrease ($p<0.05$) in the 10 μg/kg and 50 μg/kg groups as compared to control (Figure A.4), and the power was acceptable (0.991). The 100 μg/kg group was similar to the vehicle control group. There was a significant increase ($p<0.05$) in the volume density of septa in the 10 μg/kg group as compared to control (Figure A.5), although the power was low (0.631). There were no differences in the volume densities of bronchi, pleura, and vessels (data not shown). The micrographs of the tissues (20x objective) demonstrate that there were few differences between the 50 µg/kg and 100 µg/kg (Figure A.6).

Treatment	Initial Body Weight (g)	Final Body Weight (g)
Vehicle	9.8 ± 0.1	29.0 ± 2.6
20 µg/kg	9.6 ± 0.3	30.3 ± 0.2
50 µg/kg	9.8 ± 0.4	31.3 ± 1.2
100 µg/kg	9.2 ± 0.4	28.7 ± 1.0

Table A.1. Initial and final body weights from the LTD_4 concentration response.

The newborn rat pups were weighed from d4-14 PN. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no statistical differences among the groups for the initial and final days.

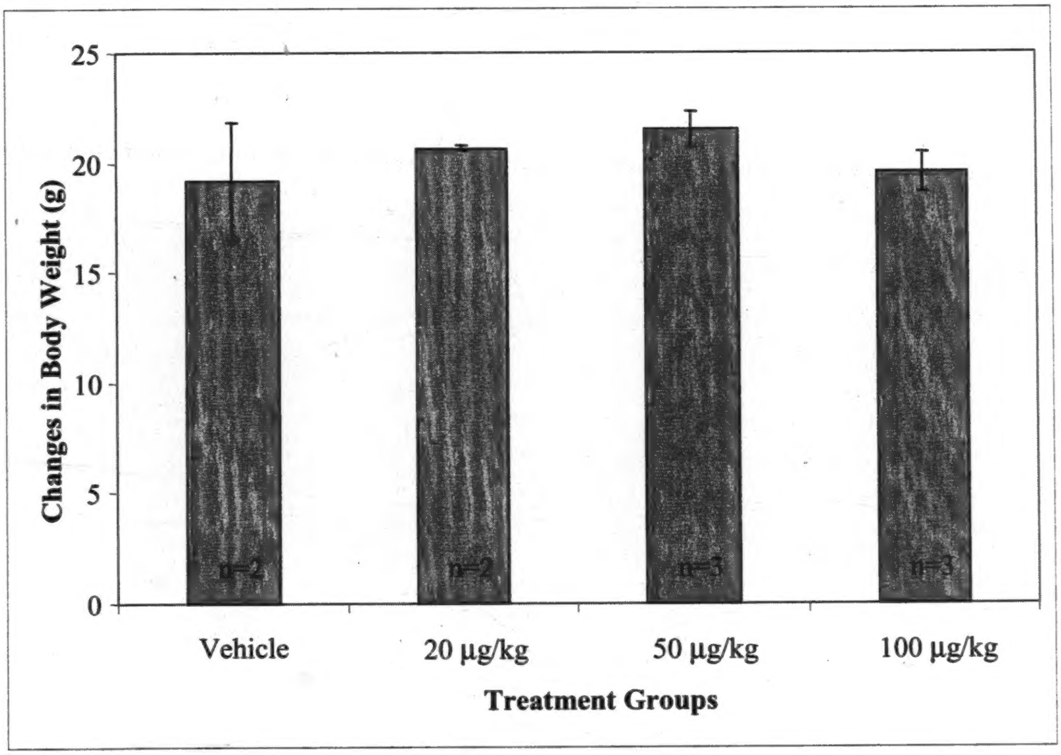

Figure A.1. Changes in body weight for the LTD_4 concentration response treatment groups.

The final body weight was subtracted by the initial body weight. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. The treatments did not cause differences in body weight as compared to control.

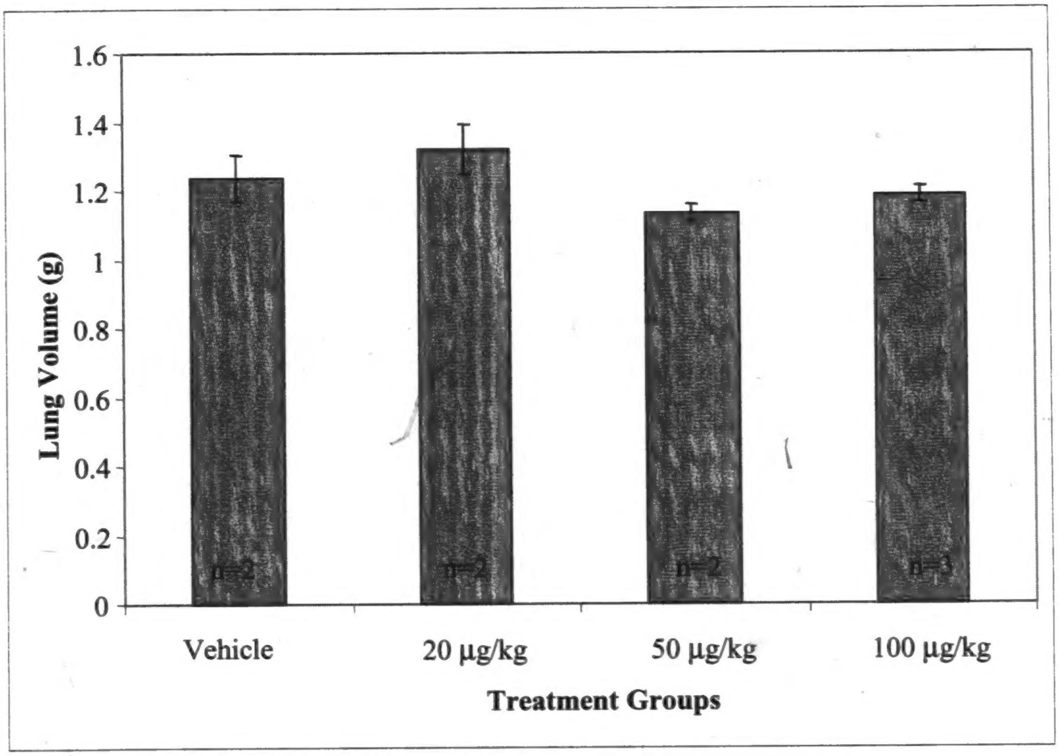

Figure A.2. Lung volumes for the LTD_4 concentration response treatment groups.

The lung volumes were determined by weighing the lungs in 4% paraformaldehyde on a balance. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There were no differences between the groups.

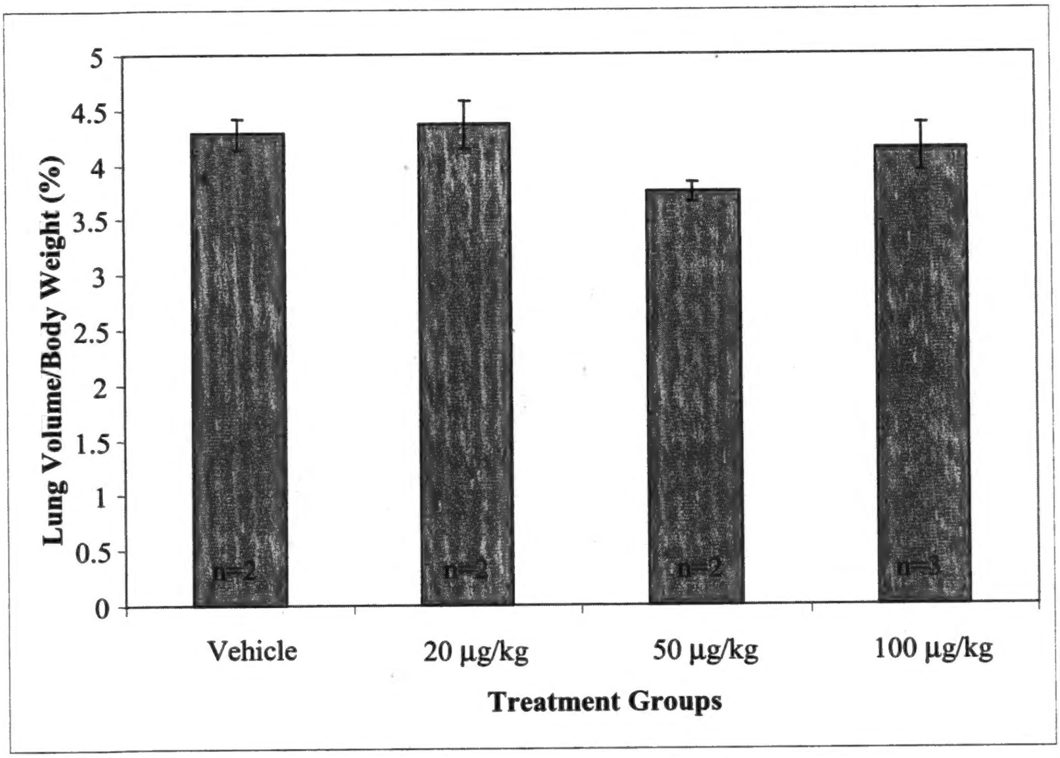

Figure A.3. **Percent body weights of the lungs (g/g BW *100) for the LTD_4 concentration response.**

The percent of the body weight of the lungs was calculated. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There were no differences between the groups.

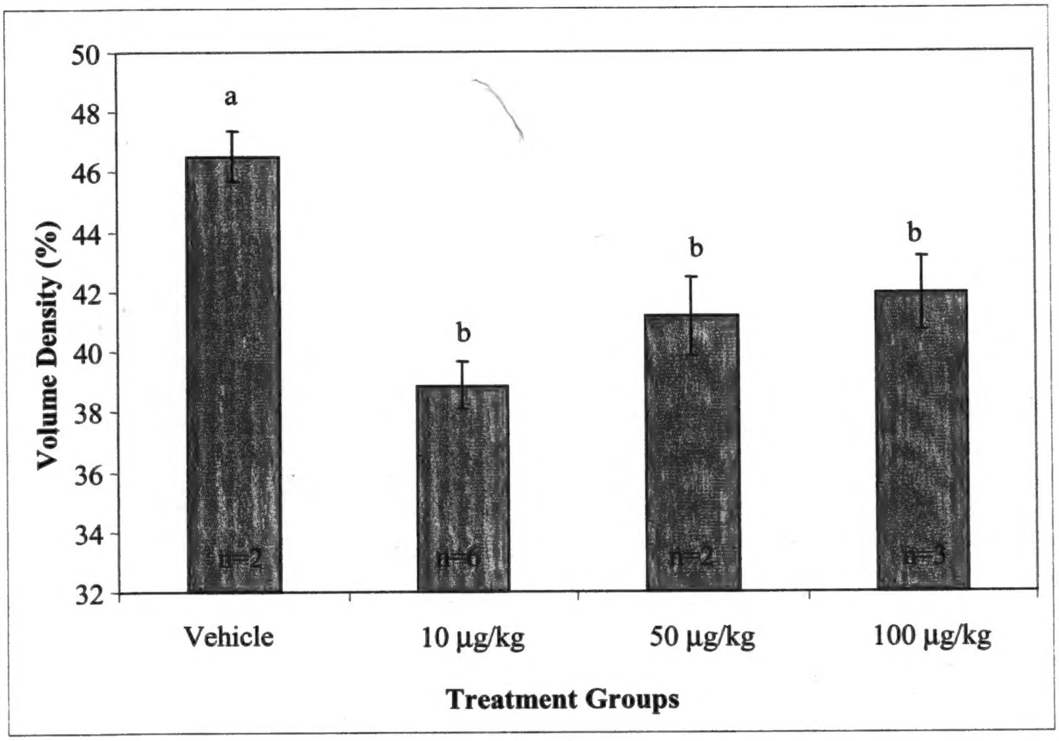

Figure A.4. Volume density of airspace for the LTD_4 concentration response treatment groups.

The measured volume density of the airspaces of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant decrease in all LTD_4 groups as compared to vehicle control.

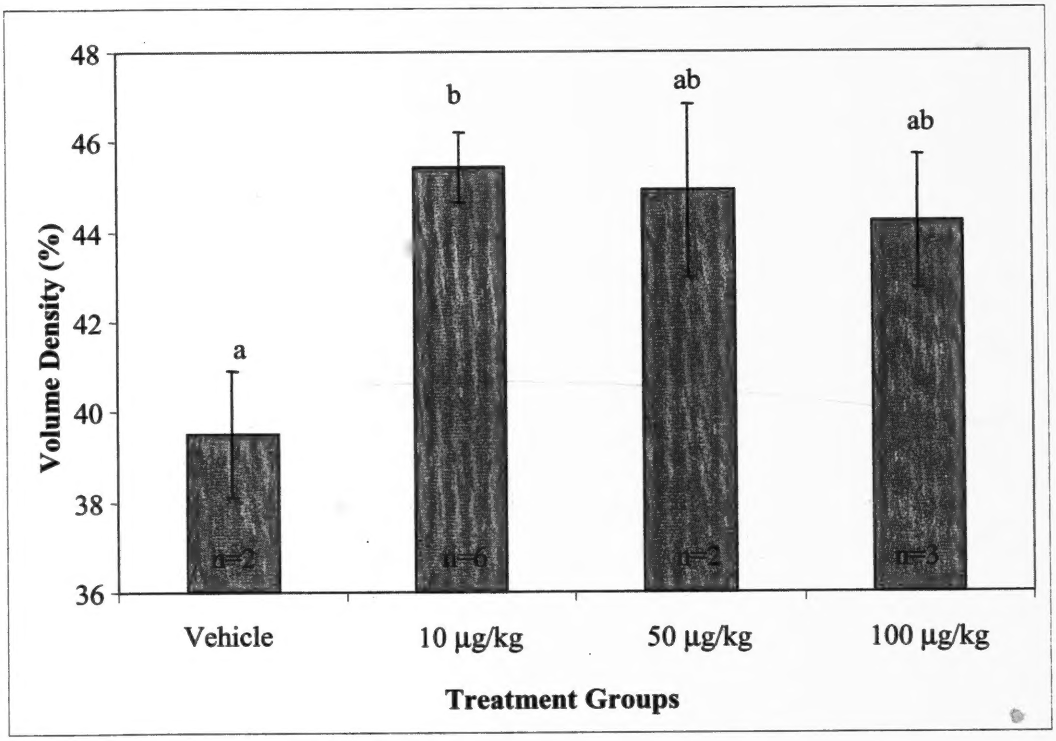

Figure A.5. Volume density of septa for the LTD_4 concentration response treatment groups.

The measured volume density of the septa of the treatment groups. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. Different superscripts represent statistically significant treatment effects ($p<0.05$), and n refers to the number of pups per group. There was a significant increase with the 10 µg/kg group as compared to control.

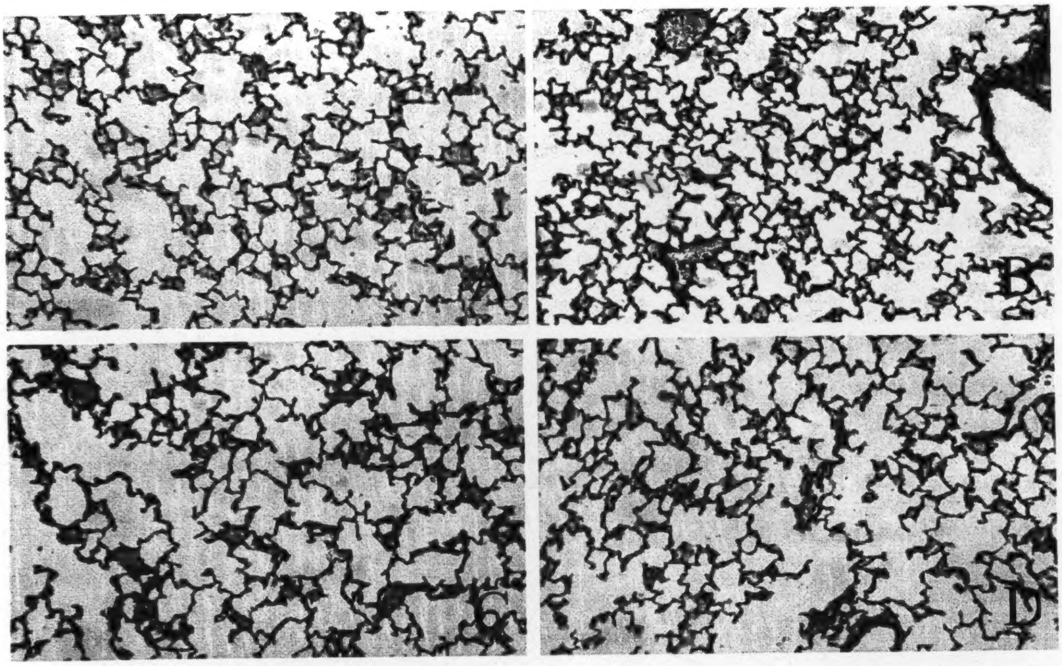

Figure A.6. Micrographs of the LTD$_4$ concentration response treatment groups using the 20x objective.

Treatment groups at the 20x objective. *A:* vehicle, *B:* 10 µg/kg; *C:* 50 µg/kg; and D: 100 µg/kg. There were few visible differences between the 50 and 100 µg/kg groups as compared to control. There was a noticeable decrease in airspace and increase in septa in the LTD$_4$ group as compared to control.

A.1.2 Discussion

The purpose of this study was to determine the optimum LTD_4 concentration that stimulated septation. This study was performed on extra rat pups from the MK-571 study; therefore, the sample size was small. The 10 μg/kg concentration caused an increase in septation, thus this study was performed as a pilot study to determine the maximal concentration of LTD_4.

The 20 μg/kg tissue was not included in the morphometric analysis. Only one tissue processed for morphometric analysis, and was not included in the analysis. The 10 μg/kg group from the previous study was included in the analysis to determine the effects of this concentration in relation to the others. There was a significant decrease ($p<0.05$) in the volume density of airspace in all three concentration groups as compared to the vehicle control group ($p<0.05$) (Figure A.5). There were no changes in the volume density of septa for the 50 and 100 μg/kg groups, whereas there was a significant increase ($p<0.05$) in the 10 μg/kg group (Figure A.6). The data suggest that the most potent concentration was 10 μg/kg, and as the concentration increased the results approached those of vehicle control.

A.1.3 Future Studies

The endpoints of these studies were the morphometric data, and not the body weight measurements. These were calculated as an afterthought to determine if there were differences; however, the sample sizes were not based on these results. Future studies should have a larger sample size to determine if the administration of exogenous LTs, at different concentrations caused differences in the data.

A.2 EFFECT OF EXOGENOUS LEUKOTRIENES ON VEGF PROTEIN LEVELS

A.2.1 Materials and Methods

An ELISA was performed to determine the amount of VEGF in the right lung samples from the organ weight study using a Quantikine M mouse VEGF Immunoassay (R&D Systems, Minneapolis, MN, USA). The samples were added to wells on a 96 well plate precoated with antibodies to VEGF. A secondary antibody was added followed by a reagent to detect the amount of bound antibody. A plate reader was used to compare the optical density of the samples to a standard curve to determine the amount of VEGF.

A.2.2 Results

The lung tissues from the organ extractions were used in an ELISA to determine if the administration of 10 µg/kg of exogenous LTs affected the levels of VEGF. There were no differences between the treatment groups and control; however, this may be due to the small sample size or to a less optimal dose of LT (Figure A.7).

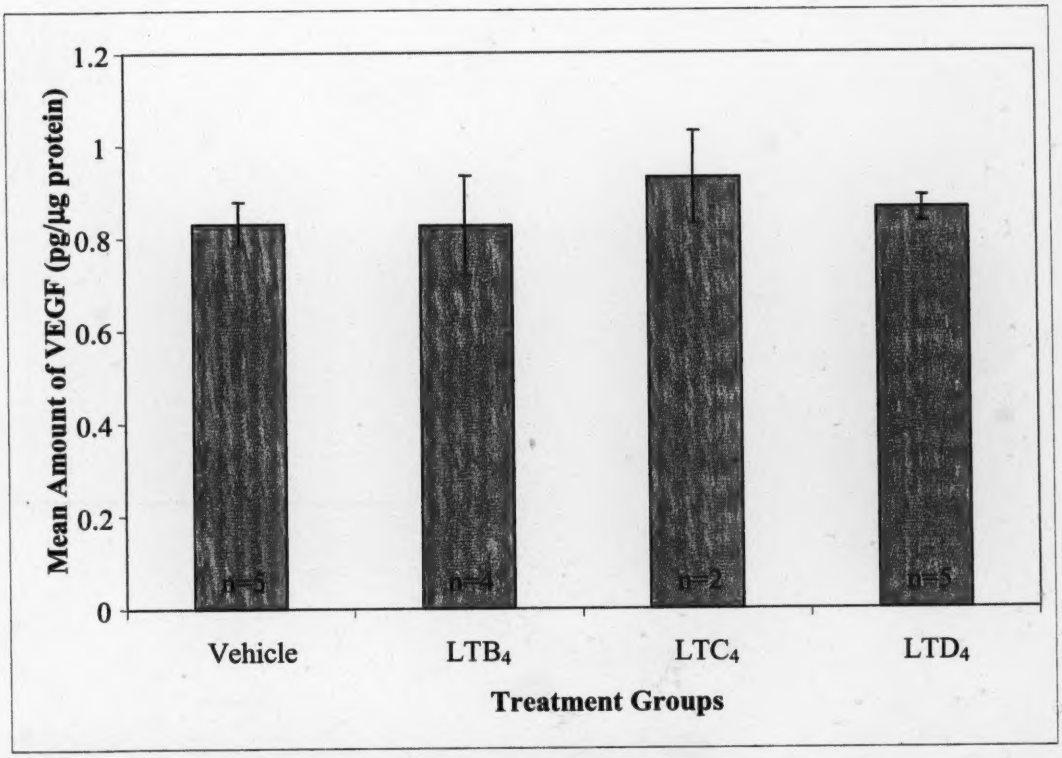

Figure A.7. Effects of exogenous LTs on VEGF levels.

ELISA to determine if the administration of exogenous LTs (10 μg/kg) caused a change in VEGF levels in the lung tissues. Data are expressed as mean ± SEM, and statistics were performed using a one-way ANOVA and Tukey's Test. There were no differences found between the treatment groups.

A.2.3 Discussion

It has been well documented that eicosanoids affect VEGF levels. Hoper *et al.* demonstrated that prostaglandins caused an increase in VEGF levels in the lung (60). An ELISA was performed to determine if the administration of 10 μg/kg of exogenous LTs would effect VEGF levels in the lung. There was a trend towards an increase between the vehicle group and the LTC_4 group. Previous studies demonstrated that VEGF decreased when endothelial cells were damaged in hyperoxia (11, 63). In the LTC_4 tissues there was an inhibition of septation and alveolarization, potentially due to damage to the endothelium. This explains why there were increased VEGF levels with LTC_4.

This study demonstrated that the administration of exogenous LTs did not change the amount of VEGF protein in the lung at this dose (Figure A.7). LTB_4 inhibits the release of VEGF, and this was not demonstrated by our data since there were no differences between the vehicle and LTB_4 groups (36). The administration of exogenous LTD_4 to human umbilical vein endothelial cells (HUVECs) did not alter the VEGF levels, which is consistent with our data (45).

A.2.4 Future Studies

This study was performed on the lung tissues collected during the organ removals. An ELISA should be performed on the concentration response tissues to examine if the various concentrations would affect the VEGF levels.

A.3 ONTOGENY STUDY OF THE CYSLT$_1$ AND CYSLT$_2$ RECEPTORS

A.3.1 Materials and Methods

A.3.1.1 Tissue Collection

An ontogeny study of the CysLT$_1$ and CysLT$_2$ receptors was begun using lung tissues collected at various days of both the embryonic and neonatal rat lung. Lungs were collected from embryos at days 18 and 20 of gestation, and at birth. They were also collected on days 4, 6, 9, 12 and 14 PN. Pups were also placed in hyperoxia and lungs were collected at days 6, 9, 12 and 14 PN.

Tissues were collected from embryos by first euthanizing the dams with a 1.5 ml intra-peritoneal injection of Euthanyl. A midline laparotomy was performed and the uterus was removed. The embryos were removed in their sacs and placed in PBS. The lungs were removed, washed in PBS, frozen in liquid nitrogen and stored at –80°C.

A.3.1.2 Western Immunoblotting

The samples were prepared so that 80 µg of protein and 6-times loading buffer were loaded per well. The samples were boiled for 5 min to ensure that the samples were properly mixed. The samples were separated on a 10% polyacrylamide gel for 2½ h in running buffer. The protein was transferred overnight from the gel onto a nitrocellulose membrane (BioRad, Mississauga, ON) in transfer buffer at 20 volts in 4°C. The membrane was stained with Ponceau-S and blocked with 7% milk for 1 h. It was washed 3-times for 15 min in TBS-0.1% Tween, and this was followed by a 2 h incubation in primary antibody. The membrane was washed 4-times for 10 min in TBS-0.1% Tween, and incubated in secondary antibody for 1 h. The membrane was washed 4-times for 10 min in TBS-0.15% Tween and rinsed in ddH$_2$O. It was developed in ECL (Western

Blotting Detection Reagent, Amersham Biosciences, Buckinghamshire, UK) for 2 min and was developed on the Fluor-S-Max (BioRad) for 30 min.

There were many problems identifying the band representing the CysLT_1 and CysLT_2 receptors. The companies that produced the polyclonal antibodies had not tested it for Western Immunoblotting. The band weights were predicted based on the amino acid sequence; however, the receptors are both glycosylated and phosphorylated (75), which would affect the weights. Also, the blocking peptides did not provide consistent results. On one gel the band would be blocked; however, the results could not be repeated. In the end these studies were put on hold due to the faulty antibodies.

A.3.2 Discussion

An ontogeny study began on tissues from both normoxic and hyperoxic animals due to the questions that arose from the data of Study 1. There was not a complete duplication of the effects of hyperoxia when exogenous LTs were administered, which may be due to insufficient levels of receptors. The hypothesis was that hyperoxia affects the levels of the CysLT_1 and CysLT_2 receptors.

The first technique used was western immunoblots. These did not provide consistent data for a variety of reasons, and there were many problems associated with identifying the bands representing the CysLT_1 and CysLT_2 receptors. The companies that produced the polyclonal antibodies had never tested them in western immunoblots. They predicted the band weight from the amino acid sequences and weights. However, the receptors were both glycosylated and phosphorylated (75), both of which would affect the weight of the protein.

The two receptors were discovered through photoaffinity labeling, which discovered two bands with weights of 66200 and 45000 daltons (104). This 45000 dalton band was thought to be the CysLT$_1$, and this was the band that was searched for on the western immunoblots. Another study confirmed that the CysLT$_1$ receptor had a molecular weight of 42 kD (46). In the rat tissues a band of this size was not detected, there were always multiple bands and the closest to this size was in the 50 kD range. This may be due to glycosylation or phosphorylation, both of which would increase the size of the band. Blocking peptides were used to identify the receptor bands; however, this did not provide consistent results. There were many bands produced, and there was a large amount of background. This indicates that the antibodies were not completely specific to the CysLT$_1$ receptor, which made it difficult to identify the band with certainty.

These studies were halted due to the uncertain antibodies. These are newly characterized receptors and the antibodies have not been completely tested. The only studies that successfully used antibodies for the CysLT receptors manufactured their own, and used them on human tissues (46). The studies will be resumed when antibodies are produced that have been tested in western immunoblotting.

A.3.3 Future Studies

When an antibody is created that has proven to be effective for both western immunoblotting and immunohistochemistry, colocalization studies must be performed. It is currently uncertain whether the airway epithelium is expressing the CysLT receptors, and it should be determined if they are.

It should also be determined if the receptors are located on the endothelium in the interalveolar capillaries. This will be done by colocalizing the receptors with von Willebrand factor. A previous study determined that this was the best marker for the endothelial cells of the fetal and neonatal pulmonary vasculature (142). Another method of localization would be to determine the cells that are expressing the mRNA of the receptors using *in situ* hybridization.

The localization of the CysLT$_1$ receptor has been limited to the cell-surface. However, recent immunohistochemistry results discovered cytoplasmic staining, suggesting that CysLT$_1$ may be located on intracellular membranes (131). Our studies on both the CysLT$_1$ and CysLT$_2$ receptors should include determining if they may also be located in the cytoplasm or on the nuclear envelope.

Recent studies determined that LTD$_4$ acts on tracheal smooth muscles cells in a manner that cannot be explained by the two characterized CysLT receptors (114). Functional studies in both human and porcine pulmonary arteries did not demonstrate the same antagonist properties as the characterized CysLT receptors (7, 8). This indicates that there may be a third CysLT receptor, and this requires further investigation.